谁言寸草心，报得三春晖

孟郊 (618-907)

爸爸妈妈，谢谢你们的养育之恩，你们的女儿，庆

KU Leuven
Biomedical Sciences Group
Faculty of Medicine
Department of development and regeneration
Stem cell biology and embryology

PROSPECTIVELY ISOLATED NGN3-EXPRESSING PROGENITORS FROM HUMAN EMBRYONIC STEM CELLS GIVE RISE TO PANCREATIC ENDOCRINE CELLS

Qing CAI

Jury:

Promoter: Prof. dr. Catherine Verfaillie
Co-promoter: Prof. dr. Chantal Mathieu
Chair: Prof. dr. Maurilio Sampaolesi
Secretary: Prof. Frederik Nevens
Jury members: Prof. Ole Dragsbæk Madsen
Prof. Leo van Grunsven
Prof. Danny Huylebroeck
Prof. dr. Frederik Nevens

Dissertation presented in partial fulfilment of the requirements for the degree of Doctor in biomedical sciences

January 2014

ACKNOWLEDGEMENTS

A PhD is a journey not a destination. By typing this down, I will be finalizing it. This PhD will guide me towards many other journeys. But before proceeding, I must thank all those people whom have encouraged and helped me along the way.

First of all, I would like to thank my promoter professor Catherine Verfaillie who has been supporting me from the moment I stepped into her lab. I have learned a lot from her during my PhD and will always think of this amazing period. Also, I have never seen a professor being so enthousiastic in a “kill-the-other-person’s-balloon” game, thumbs up!

I am also grateful for all the members of the jury: prof. Ole Dragsbæk Madsen, prof. Leo van Grunsven, prof. Danny Huylebroeck, prof. dr. Frederik Nevens for the time devoted in reviewing my thesis and for giving comments and helpful suggestions. Also, I would like to thank prof. Maurilio Sampaolesi for being the chair of my committee and for all the small (and big) talks we had when passing by. Lots of love for your cute family!

Next, I would also like to thank dr. Laura Ordovas as she has been my guide during the last years of my PhD. Like a big sister she looked over my shoulder and shook me from time to time to alert me when I was deviating. Many thanks for the help and support! I hope you can finally settle down in Spain soon. I will try to pursue Imec to start a life science department in Zaragoza. In my very last year, I also had the chance to work closely with dr. Paola Bonfanti for which I am very grateful. I enjoyed very much our interaction and discussions. And I wish all the best for you and your future career in London!

For sure, I also have to thank my family. My parents showed me that you can get far as long as you have the courage and belief. By setting up their own business, they were able to give us a head start in live. A million thanks goes out to my beloved sister Hua, Luc and their kids. Especially my sister Hua has been a tremendous help with everything (styling issues, Z4, birth cards, covers, invitations, taoboa orders, clothing…) whenever and where ever. She has set the pace for all of the siblings, which I admire greatly. Also, my big brother Jun has been very caring especially during my masters in Leuven by opening a cool wok restaurant (Wok on Air) where I could eat every day for free. Next, I would also like thank my family in law, especially Luk and

Daisy for their time and patience with me and my little family. I could not wish for better parents-in-law.

I also would like to thank Julie, Sanne, Stijn, Cedric, Jeroen en Daphné for being my closest friends. We haven't seen each other very often due to our busy agenda's, but the moments spent were worthwhile.

I cannot forget to thank all lab members from the 5th floor for all the nice side activities and relaxing atmosphere. Especially, Kim, Tineke and Susanna were always in for fun discussions and jokes. You go girls!!! Also, Kristel, Veerle, Ranga, Jos, Vik and Pieter helped me a lot and I will always be grateful for that. I would also like to acknowledge Christina for her help with my administration and the good advice she gave me. Special thanks for Jolien for being my lab mate. In our lab, most people spent their entire PhD in the dreadful cell culture hood, but thanks to Jolien it was really fun. I will miss our discussions, but luckily machines called phones exist. Although, the disadvantage is the look on the faces of our significant others each time we spent several hours on it.

Probably, the biggest thank you goes out to my significant other, Marjolijn, as she has always stood beside me. For all those times spent home alone, later on with one and even more later on with two "energy-consuming" kids during the week-ends and evenings, when I had to be in the lab. Dank u, liefje! Jij bent een onmisbare grote schat naast mijn andere twee kleine schatjes. Off course, I also have to thank little cute Laura and Noor for demanding so much attention in a way I forget about all other things. Little cute Laura will for sure miss the parties at mamies "old" work as she enjoyed very much playing with the person sharing the same name.

I hope I did not forget anyone! Thank you for sharing this day with me. All the best to you and maybe we will see each other again!

ABSTRACT

Pancreatic endocrine progenitors obtained from human embryonic stem cells (hESCs) could be used as an alternative source to develop cell-based therapies for diabetes mellitus (DM). However, in human a true endocrine progenitor has not been isolated yet. Therefore, the aim of our study is to investigate whether it is possible to isolate pancreatic endocrine progenitors from differentiating hESC cultures by lineage tracing of NGN3, a known marker of mouse pancreatic endocrine progenitors. We targeted the 3' end of *NGN3* using zinc finger nuclease-mediated homologous recombination to allow selection of NGN3eGFP$^+$ cells from hESCs differentiating towards a pancreatic fate without disrupting the *NGN3* gene. These NGN3eGFP$^+$ cells express PDX1, NKX6.1 and chromogranin A, and differentiate *in vivo* towards insulin, glucagon and somatostatin single hormone-expressing cells, but not to ductal or exocrine pancreatic cells or other endodermal, mesodermal or ectodermal lineages, confirming our hypothesis that NGN3$^+$ cells represent pancreatic endocrine progenitors in human. Overall, this hESC reporter line can aid in gaining insights in developmental mechanisms underlying fate choices in human pancreas as well as in generating cell-based therapies.

SAMENVATTING

Het gebruik van pluripotente stamcellen voor de regeneratieve geneeskunde is veelbelovend, omdat deze cellen voorzien zijn van enorm potentieel voor celtherapie alsook voor het onderzoeken van de ontwikkeling van organen en ziektes zoals diabetes mellitus (DM) type 1. Een ziekte dat wordt gekenmerkt door een verhoogde glucoseconcentratie in het bloed, te wijten aan de afwezigheid van β-cellen in de pancreas. Deze cellen zouden kunnen vervangen worden door β-cellen afkomstig van gedifferentieerde pluripotente stamcellen. Op deze manier, zouden patiënten kunnen verlicht worden van hun dagelijkse insuline-injecties. De meeste studies waarin pluripotente stamcellen worden gebruikt voor het genereren van β-cellen maken gebruik van gemengde of verrijkte celpopulaties. In die zin, is het moeilijk af te leiden welke cel werkelijk bijdraagt tot de gewenste β-cellen. Daarom is een betrouwbare en nauwkeurige techniek nodig voor het nagaan welke cellen verantwoordelijk zijn voor het genereren van de cellen van de eilandjes van Langerhans. Hiervoor maakten we de hypothese, dat het mogelijk is om humane endocriene pancreas voorlopercellen te selecteren door middel van de specifieke selectie van neurogenin-3 (NGN3) expresserende cellen afkomstig van gedifferentieerde humane embryonale stamcellen (hESC). Een alternatieve bron voor β-cellen zou op deze manier kunnen gegenereerd worden. In de muis werd al aangetoond dat Ngn3-expresserende cellen kunnen differentiëren naar alle pancreatisch endocriene cellen: α-, β-, δ-, ε- and PP-cellen. Daarom is Ngn3 een nuttige merker voor cellen die van een progenitorrol verder differentiëren naar een meer matuur endocriene rol. Om de selectie van Ngn3-expresserende cellen mogelijk te maken, hebben we gebruik gemaakt van de “zinc finger nuclease” (ZFN) gemedieerde homologe recombinatie (HR) technologie voor het genereren van hESC lijnen waarbij een fluorochroom tot expressie komt, wanneer *NGN3* wordt geïnduceerd. Dit gebeurt zonder dat de coderende sequentie van het NGN3 gen wordt aangepast. Dankzij de differentiatie van deze reporterlijnen was het mogelijk om >90% zuivere NGN3eGFP$^+$ cellen te isoleren. Na transplantatie (zonder voorafgaande celkweek) van deze opgezuiverde humane NGN3eGFP$^+$ cellen onder het nierkapsel van gezonde muizen, konden we de cellen verder differentiëren in pancreatisch endocriene cellen, meerbepaald in insuline-, glucagon- en somatostatine-expresserende cellen. Dit is bijgevolg het eerste gedetailleerde verslag van een knock-in add-on in een gen, dat niet tot expressie komt in hESC. Deze gegenereerde cellijn is uitermate geschikt voor “lineage tracing” studies, die kunnen

helpen bij het verkrijgen van meer inzicht in ontwikkelingsmechanismen, die aan de basis liggen van celkeuze beslissingen alsook voor het genereren van celgebaseerde therapieën.

SUMMARY

The use of pluripotent stem cells for regenerative medicine holds great promise, because these cells are foreseen to have a tremendous potential in cell-replacement therapies as well as in elucidating developmental and disease pathways. Therefore a lot of effort has been put in exploring their ability to treat diabetes mellitus (DM) type 1, which is characterized by elevated blood glucose levels due to the absence of β-cells in the pancreas. Replacing these β-cells with pluripotent stem cell-derived β-cells could relief the patient from his daily insulin injections. Most studies using pluripotent stem cells for recreating β-cells directly or through the generation of pancreatic progenitors rely on the transplantation of mixed or enriched cell populations. In this sense, the understanding of which cell contributes to the derivation of the desired β-cells remains unclear. Therefore, a reliable and precise tool is needed to track the cells responsible for regenerating pancreatic islet cells. For this purpose, we hypothesized that it might be possible to select pancreatic endocrine progenitors by specific selection of neurogenin-3 (NGN3)-expressing cells from differentiating hESCs which could be used as an alternative cell source to generate β-cells. In the mouse, Ngn3 expressing cells are known to give rise to all hormone-secreting pancreatic endocrine cell types. Therefore, Ngn3 should be a useful marker for cells that are transitioning from a pancreatic epithelial progenitor cell fate to an endocrine progenitor cell fate. In this light, we made use of the zinc-finger nuclease (ZFN) mediated homologous recombination (HR) technology to generate hESC lines wherein a drug resistance and fluorochrome selectable cassette has been knocked in using at the 3'end of the *NGN3* locus, without disrupting the coding sequence of the *NGN3* gene. Differentiating these targeted hESCs made it possible to select for >90% pure $NGN3^+$ cells from mixed hESC progeny. Upon transplantation (without pre-cultivation) of these purified human $NGN3^+$ cells under the kidney capsule of healthy mice, we could direct these cells further towards endocrine hormone-expressing cells, more specifically to insulin, glucagon or somatostatin expressing cells. This represents the first detailed report of a knock-in add-on into a non-expressed gene in hESCs suitable for lineage tracing studies, which can aid in gaining insights in developmental mechanisms underlying fate choices as well as in generating cell-based therapies.

Acknowledgements i

Abstract iii

Samenvatting iv

Summary vi

List of abbreviations x

1 Introduction 1

1.1 Diabetes Mellitus 2

a. Description 2

b. Types of diabetes mellitus 2

c. Symptoms and complications 4

d. Treatment of diabetes 5

1.2 Pancreas development 7

1.3 Histology of the pancreas 11

1.4 Pancreatic transcription factors 13

1.5 Replication and neogenesis of β-cells 16

a. Replication of pre-existing β-cells 16

b. Neogenesis of β-cells 18

1.6 Alternative cell sources for β-cell therapy 19

a. Reprogramming towards β-cells 19

b. Differentiation of other adult non-β-cell sources 22

1.7 Genome editing of PSCs 27

2 Aims 30

2.1 Aim 1: Genetically modify hESCs to generate a reporter cell line for *NGN3* 31

2.2 Aim 2: Characterization of the prospectively isolated NGN3eGFP^{+} cells 31

2.3 Aim 3: Assessing the potential of the NGN3eGFP$^+$ cells 31
4 Materials and methods 32
4.1 Cell culture 32
4.2 RNA isolation and quantitative real time-polymerase chain reaction (qRT-PCR) 33
4.3 NGN3 gene targeting 33
4.4 PCR genotyping 34
4.5 Teratoma formation and analysis 35
4.6 Southern blot 35
4.7 Array Comparative Genomic Hybridization (aCGH) and karyotyping 35
4.8 Immunocytochemistry of NGN3eGFP$^+$ cells 36
4.9 Fluorescence-activated cell sorting (FACS) 36
4.10 Immunohistochemistry and FISH 37
4.11 *In vitro* c-peptide production 38
4.12 Transplantation under the kidney capsule 38
4.13 Statistical analyses 38
4.14 Tables 39
5 Results 42
5.1 Generation of NGN3eGFP-hESC lines 42
5.2 Differentiation of hESCs towards endocrine pancreas 47
5.3 Characterization of the NGN3eGFP$^+$ cells from differentiating hESCs 54
5.4 NGN3eGFP$^+$ cells have the capacity to differentiate towards endocrine hormone-expressing cells *in vivo* 58
6 Discussion 60
7 Conclusion and future perspectives 63

8 Referenties 66

9 Appendix 81

10 Curriculum vitae 84

LIST OF ABBREVIATIONS

DM: diabetes mellitus

BC: before christ

β-cells: beta-cells

IGT: impaired glucose tolerance

IFG: impaired fasting glucose

GDM: gestational diabetes mellitus

MODY: maturity-onset diabetes of the young

HNF: Hepatocyte nuclear factor

Pdx1: pancreatic and duodenal homeobox 1

DP: dorsal bud

VP: ventral bud

α-cells: alpha-cells

δ-cells: delta-cells

ε-cells: epsilon cells

PP-cells: pancreatic polypeptide cells

TF: transcription factor

Ptf1a: pancreas transcription factor 1a

Sox: SRY (sex determining region Y)-box 9

Ngn3: Neurogenin3

NeuroD1: Neurogenic differentiation factor 1

Pax4: Paired box 4

Arx: Aristaless related homeobox

Nkx2.2: NK2 homeobox 2

Nkx6.1: NK6 homeobox 1

HPAP: human placental alkaline phosphate

BrdU: bromodeoxyuridine

GLP-1: glucagon-like peptide 1

EMT: epithelial-to-mesenchymal transition

Ins: insulin

HDAD: helper-dependant adeno-viral vectors

Psck: Prohormone convertase

Kir6.2: Potassium inwardly rectifying channel, subfamily J, member 11

Abcc8: ATP-binding cassette, sub-family C, member 8

BTC: betacellulin

DPPIV: dipeptidyl peptidase IV

GCK: glucokinase

PC1/3: prohormone convertase 1/3

GFP: green fluorescent protein

CMV: cytomegalovirus

Tuji: Neuronal class III β-tubulin

Cpa1CreER^{T2}: CarboxypeptidaseA1, pancreatic, targeted mutation 1

MafA: v-Maf musculoaponeurotic fibrosarcoma oncogene homolog A (avian)

Hnf6: Hepatocyte nuclear factor 6

BM: bone marrow

MSC: mesenchymal stem/stromal cells

PSCs: human pluripotent stem cells

ESCs: embryonic stem cells

iPSCs: induced pluripotent stem cells

SSEA: stage-specific embryonic antigen

OCT4: octamer-binding transcription factor 4

KLF4: Krupple-like factor 4

cMYC:c-myc myelocytomatosis viral oncogene homolog (avian)

GT: gut tube

AF: anterior foregut

Wnt3a: wingless-type MMTV integration site family, member 3a

SHH: sonic hedgehog

RA: retinoic acid

BMP: bone morphogenetic

IDE: induce definitive endoderm

ILV: indolactam V

HR: homologous recombination

ZFN: zinc finger nuclease

TALEN: transcription activator-like effector nuclease

NKX2.5: NK 1 homeobox 5

GCG: glucagon

TRK: tropomyosin-related kinase

ROCK: Rho-associated kinase

Dsb: double-strand break

NA: nicotinamide

BSA: bovine serum albumin

IGFII: insulin-like growth factor II

qRT-PCR: quantitative real-time polymerase chain reaction

FISH: fluorescent *in situ* hybridization

PBS: phosphate buffered saline

FACS: fluorescence-activated cell sorting

KRBH: Krebs-Ringer solution with bicarbonate and HEPES

eGFP: enhanced green fluorescent protein

Puro^{R}: puromycin resistance

EF1α: Elongation Factor 1 alpha

Hyg/TK: hygromycin^{R}/thymidine kinase

WT: wild type

PS: primitive streak

EOMES: eomesodermin

MIXL1: Mix1 homeobox-like 1

CXCR4: C-X-C chemokine receptor type 4

FOXA2: forkhead box protein A2

HLXB9: homeobox gene HB9

CHGA: chromograninA

SST: somatostatin

IBMX: phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine

CK19: cytokeratin 19

GFAP: glial fibrillary acidic protein

CDX2: caudal type homeobox 2

LSSC: low side scatter

HSSC: high side scatter

1 INTRODUCTION

The term diabetes mellitus (DM) has both Greek and Latin roots. Diabetes originates from the Greek word for siphon, alluding to one of the symptoms of the disease concerning the excessive water intake which is immediately lost as if it had run through a water pipe, while Mellitus is Latin for honey, describing the sweet taste of diabetic urine. The disease, DM, was described for the first time in the 3rd century BC by the Greek physician, Demetrius of Apameia, and it was not until 1922 that the first patient was treated with insulin[1].

DM which previously has been linked to rich nations has now evolved towards a pandemic. The International Diabetes Federation estimates that by 2030 approximately 366 million people worldwide will be suffering of DM. DM is characterized by continued elevated levels of blood glucose either due to the loss of insulin producing β–cells in the pancreas (type 1), or due to decreased sensitivity to insulin (type 2)[2]. Type 1 DM can be treated with insulin injections to normalize blood glucose levels. However, patients still suffer from hyper- and hypoglycemia with life-threatening complications such as myocardial infarcts, peripheral limb ischemia and stroke. Novel routes of insulin administration e.g. intranasal administration, inhaled insulin, gastrointestinal delivery are being examined[3]. For patients with type 1 DM, and to a lesser extent for type 2 DM, the only curative therapy is replacement of lost β–cells by grafting whole pancreas or islets[4]. However, as several cadaveric pancreata are needed to obtain sufficient islets to treat a single patient, shortage of donor pancreata prevents the use of this therapy in large groups of patients. In addition, although islet grafts can control the glycemia of patients for a period of a few years, loss of the graft with time may necessitate the transplantation of islets on multiple occasions[5]. Hence the need for alternative, and if possible renewable, sources of β–cells.

1.1 **Diabetes Mellitus**

a. Description

β-cells play a pivotal role in controlling blood glucose homeostasis. Most of the food we consume is broken down or metabolized into the monosaccharide glucose. The body utilizes glucose as the main source of fuel. After digestion of food, glucose enters into the bloodstream, where it is used by cells to sustain proliferation and metabolic functions. In order for glucose to enter into cells, insulin is required. Insulin is a hormone produced by the pancreas, a large gland located behind the stomach. When we eat, the appropriate amount of insulin is normally produced by the pancreas in order to move glucose from the blood into cells. However, people with DM have problems either with insulin production or insulin resistance (i.e. cells are incapable of reacting to the insulin that is produced). As a consequence, the glucose concentration in the blood rises (hyperglycemia), and is excreted in the urine. Thus, large concentrations of glucose cannot be used as a source of fuel for the body, and the body starts to use fat to support cell metabolism and proliferation. Breakdown of fat tissue results in the production of ketones, and ketoacidosis is often seen in patients with untreated DM and hyperglycemia[6]. Therefore, DM is described as a metabolic disorder of multiple etiologies that is characterized by continually elevated levels of blood glucose or by transitory inadequate utilization of ingested glucose. DM can be distinguished from the pre-diabetic states, impaired glucose tolerance (IGT) and impaired fasting glucose (IFG). DM is caused by absolute or relative insulin deficiency. This disease has many health complications, including coronary heart disease, stroke, peripheral vascular disease, blindness, and kidney disease among others[2].

b. Types of diabetes mellitus

DM can be classified into four major groups: type 1 DM, type 2 DM, gestational DM and other types (Table 1). Type 1 arises from selective, irreversible destruction of insulin-producing pancreatic β-cells. There are two sub-types of type 1 DM: immune-mediated type 1A DM and idiopathic type 1B. Type 1A DM is characterized by the fact that the immune system attacks and destroys the insulin-producing β-cells in the pancreas. The pancreas then produces little or no insulin. Type 1B DM has no known etiologies and is idiopathic. This form of DM is strongly

inherited, but currently lacks immunological evidence for β-cell autoimmunity. A patient with type 1 DM must take insulin daily to live[2, 7].

The most common form of DM is type 2 DM. This form of DM is most often associated with older age, obesity, family history of gestational DM, physical inactivity, and certain ethnicities. Type 2 DM is characterized by insulin resistance, where insulin is not efficiently used or is produced in insufficient amounts[6]. Unlike type 1 DM, which always requires insulin therapy, type 2 DM treatment options include behavioral modification and administration of various oral anti-diabetic agents[2].

The third form of diabetes is called gestational diabetes mellitus (GDM) and usually manifests itself in the third trimester of pregnancy [2]. GDM is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. In the majority of cases of GDM, glucose regulation will return to normal after delivery[7].

Other types of DM, reported by the Expert Committee on the diagnosis and classification of DM include less common types of largely unknown etiology[2, 7]. For example, maturity-onset diabetes of the young (MODY) are caused by genetic defects in the genes encoding one of the transcription factors *HNF4α*, *HNF1α*, *HNF1β*, *PDX1* and *GLUCOKINASE*. This heterogeneous subgroup affects approximately 2% of all diabetic patients and affects 2-5% of patients already diagnosed with type-2 DM. Identification of the genetic basis for this type of DM has provided not only valuable information into the pathology of those afflicted, but has also increased our basic understanding of glucose homeostasis and has defined potential therapeutic targets[2, 8].

Table 1 Etiologic classification of DM**, adapted from Gavin *et al.* 2003.**

I. Type 1 DM*** (β-cell destruction, usually leading to absolute insulin deficiency)**

A. Immune mediated

B. Idiopathic

II. Type 2 DM*** (may range from predominantly insulin resistance with relative insulin deficiency to a predominantly secretory defect with insulin resistance)**

III. Gestational diabetes mellitus (GDM)

VI. Other specific types

A. Genetic defects of β-cell function

B. Genetic defects in insulin action

C. Diseases of the exocrine pancreas

D. Endocrinopathies

E. Drug- or chemical-induced

F. Infections

G. Uncommon forms of immune-mediated DM

H. Other genetic syndromes sometimes associated with DM

***Patients with any form of DM may require insulin treatment at some stage of their disease. Such use of insulin does not, of itself, classify the patient.**

c. Symptoms and complications

Symptoms of type 1 DM usually develop over a short period, although β-cell destruction can begin years earlier. Symptoms may include increased thirst and urination, constant hunger, weight loss, blurred vision and extreme fatigue. If not diagnosed and treated with insulin, a person with type 1 DM can lapse into a life-threatening diabetic coma, also known as diabetic ketoacidosis. The symptoms of type 2 DM develop gradually. Symptoms may include fatigue, frequent urination, increased thirst and hunger, weight loss, blurred vision, and slow healing of wounds. Some people have no symptoms[6].

Several complications are known to be caused by DM: microvascular complication, macrovascular complications, diabetic foot disorder and dermatologic disorders. Microvascular complications include: retinopathy, nephropathy and neuropathy. A continuous relation exists

between glycemic control and the incidence and progression of microvascular complications[9, 10]. A subset of patients with DM suffers from macrovascular complications such as atherosclerotic disease with death as a consequence. Important risk factors for atheromatous large vessel disease in patients with DM are e.g. smoking, elevated blood pressure, proteinuria, and elevated cholesterol levels. Hypertension affects at least half of the patients with DM and as a consequence, these patients will eventually suffer from coronary heart disease, peripheral vascular disease, stroke, and/or erectile dysfunction[9]. Foot disorders are a major source of morbidity and a leading cause of hospitalization for patients with DM. Significant complications of the disease are ulceration, infection, gangrene, and amputation. Charcot foot, which in itself can lead to limb-threatening disorders, is another serious complication of long-standing DM[11]. It is estimated that up to 70% of DM suffer from pathologic skin changes during the course of the disease. Onset can already occur in patients with pre-DM (IGT and IFG). No skin disease is specific to DM[2].

d. Treatment of diabetes

Type 1 diabetes

The treatment of patients with type 1 DM is currently limited to insulin injections. Conventional insulin is of porcine or bovine origin, but use of these insulins is associated with eventual immune reactions against the xenogeneic protein resulting in insufficient absorption, and delayed and decreased blood peak levels. Most of the types of conventional insulin have now been replaced by recombinant human insulin. Several forms of insulin exist: short-acting, intermediate-acting and long-acting insulin analogs. Novel routes of insulin therapy are being developed to help overcome the practical difficulties of working with injection needles, such as intranasal administration, inhaled insulin, and gastrointestinal delivery[3].

Type 2 DM

Type 1 DM is currently only treated with insulin application, whereas type 2 DM patients have more treatment options. Lifestyle modification is recommended and oral anti-hyperglycaemic

agents can be administered. Several classes of oral anti-hyperglycaemic agent are known: α-glucosidase inhibitors of intestinal carbohydrate absorption, biguanides to target hepatic insulin resistance, insulin secretagogues to increase insulin secretion, insulin sensitizers or thiazolidinediones to target adipocyte and muscle insulin resistance, and intestinal lipase inhibitor or orlisat to inhibit fat absorption and promote weight loss in obese patients[12].

1.2 **Pancreas development**

The development of the pancreas has been extensively studied in mice and is a complex process. The pancreas arises from the endoderm germ layer, consisting of a two-dimensional sheet of cells, which can be found at the edge of the embryo (as shown in Figure 1a). The hindgut and foregut are formed by folding the endodermal epithelium anteriorly and posteriorly, respectively. In this way, the gut forms a tube by closing it[13]. Numerous studies have shown that the formation and differentiation of the pancreas from gut endoderm requires a series of distinct signals to form two independent thickenings called the ventral and the dorsal pancreas. Since, particular regions of the gut endoderm are competent to develop into pancreatic cells, additional extrinsic signals from adjacent tissues are still needed for the growth and differentiation into pancreatic cells[14]. These regions can be found immediately posterior to the anlagen of the stomach[15].

The dorsal and ventral pancreatic thickenings are not completely identical and develop in an asynchronous fashion. This is due to the fact that each part receives a distinct set of signals from their immediate surrounding tissues as shown in Figure 2. The dorsal pancreatic bud develops in close proximity to the notochord. The presence of the notochord near the dorsal bud is essential for its development, particularly in the beginning[16-18]. Later, the notochord will be displaced by the dorsolateral splanchnic mesenchyme, which forms the dorsal aorta located between the dorsal epithelium and the notochord. This results in a new set of signals produced by the aorta that affect the pancreas. The ventral bud of the pancreas lies near the liver and bile duct epithelium that forms on the ventral face of the gut. Organogenesis of the liver and the ventral pancreatic bud are closely related. The liver is specified by instructive signals from the cardiac mesoderm. In the absence of these instructive signals, the ventral pancreatic program is initiated. The ventral pancreatic fate program appears to be a default state, as absence of cardiac induction specifies the foregut endoderm in the prospective liver domain to a ventral pancreatic fate[15, 19].

The dorsal pancreatic bud will first arise on the opposite side of the gut tube after which the ventral bud arises immediately adjacent to the hepatic diverticulum. As the stomach and the intestine rotate, the ventral bud and hepatopancreatic orifice will move along until they fuse with the dorsal bud, which is also facilitated by growth of the buds. The ventral bud forms the posterior part of the head, or uncinate process, while the dorsal bud forms the remainder of the

organ. The ventral duct fuses with the distal part of the dorsal duct to become the main pancreatic duct[16]. Figure 1 shows the sequence of events that take place during pancreatic organogenesis.

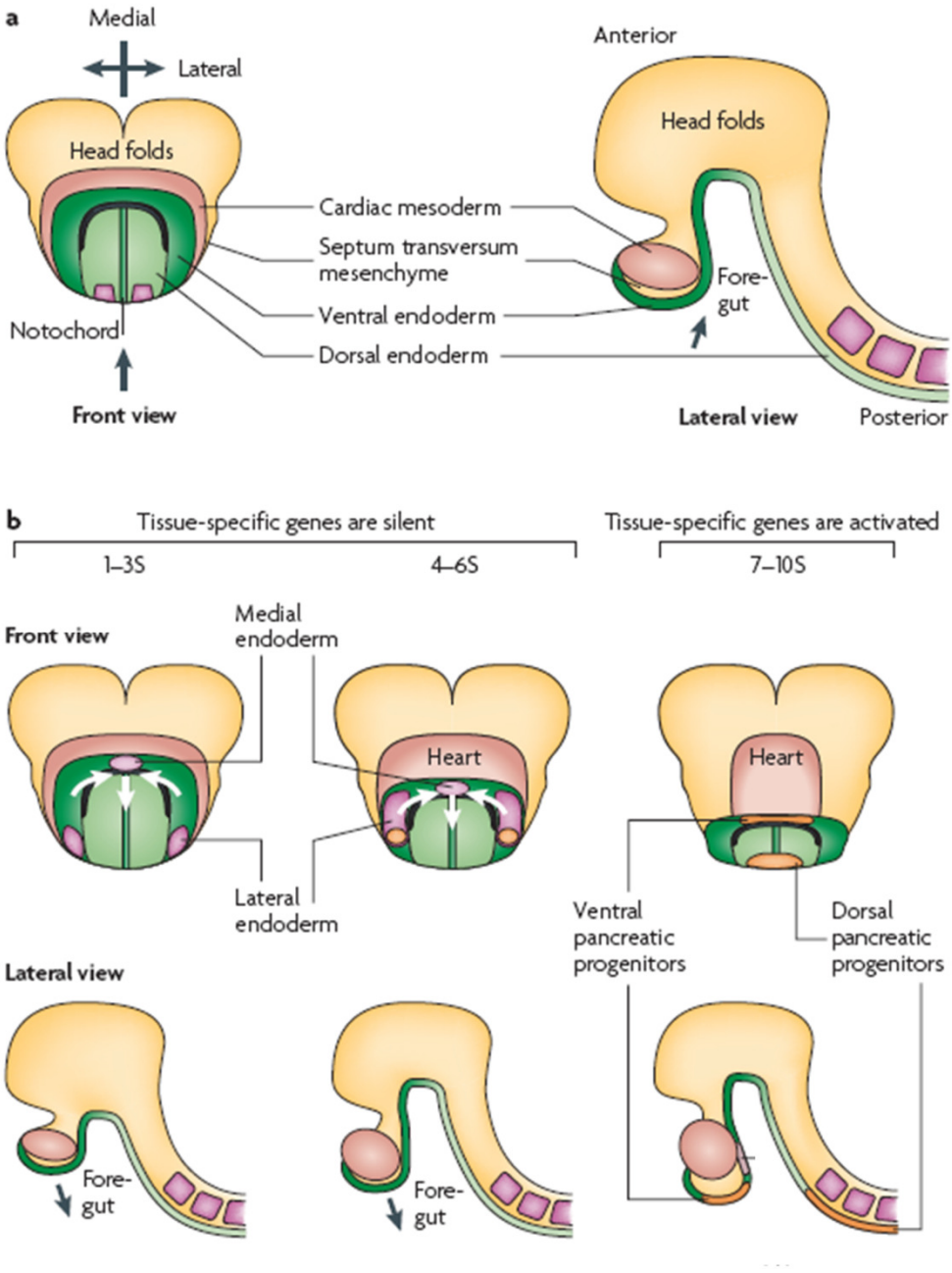


Figure 1 The development of the pancreas. (a) Front and lateral view of the anterior portion of a mouse embryo at 8.25 days of gestation. (b) Fate map of pancreatic domains in the mouse (also front and lateral view) but at different somites of development. The pancreas is specified within the endoderm by the 7S stage, whereupon the genes hat are specific to those lineages can be first detected to be active in the respective progenitor-cell populations. The pancreatic progenitors (orange) reside in paired lateral domains of endoderm (the ventral pancreas) and a medial dorsal domain (the dorsal pancreas). Arrows indicate the direction of movement of cells in designated tissues. (taken and adapted from Zaret, 2008)

Most cells composing the ventral and the dorsal bud are apparently uncommitted progenitor cells, because they have the capacity to differentiate into any of the mature pancreatic cell types. The first differentiated cells that can be detected in the developing buds are the glucagon expressing α-cells, followed by the insulin-producing β-cells, somatostatin-producing δ-cells and pancreatic polypeptide-producing PP-cells. These cells first appear at or close to the ducts, they subsequently aggregate and form islet structures late in fetal development[20].

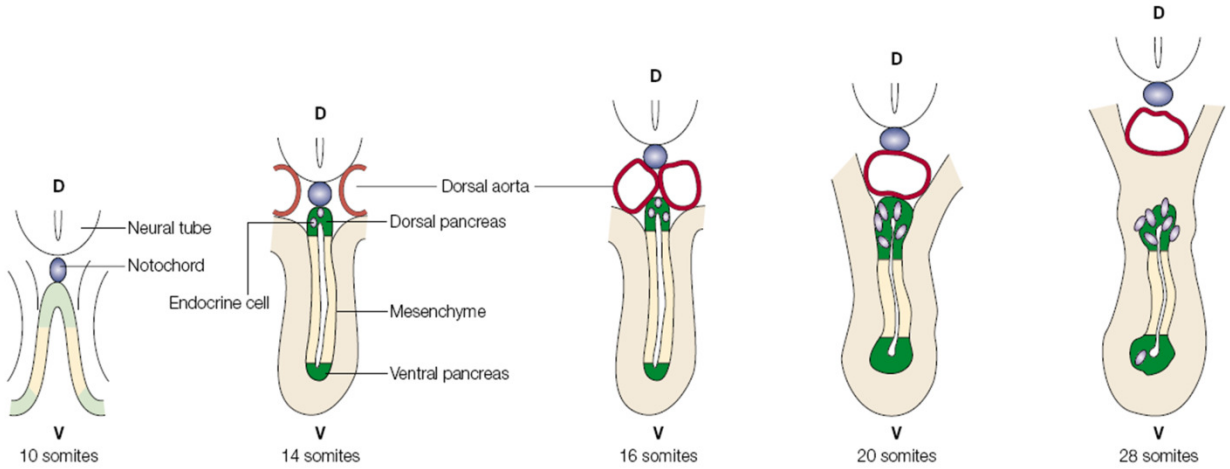


Figure 2 Early stages of pancreatic organogenesis. Cross sections (illustrations) through a mouse embryo at the level of the developing pancreas are shown. The prospective pancreatic portion (green) of the developing gut endoderm is specified before the ten-somite stage. The first endocrine cells (purple) appear by the 14-somite stage. Tissues such as the notochord (blue), endothelium (red) and mesenchyme (yellow) support pancreatic development. The ten-somite stage roughly corresponds to embryonic day (E)8, whereas the 28-somite stage roughly corresponds to E10. D, dorsal; V, ventral (taken from Edlund 2002).

The observations described above were mainly performed in rodents. Therefore, one needs to be cautious in extrapolating the data to human as not all developmental mechanisms are conserved amongst species. The few studies performed in humans involved immunohistological analyses[21,

[22], gene expression profiling[23, 24] and transplantation experiments[25, 26] of fetal pancreatic tissue of no less than 7 weeks and up to 23 weeks of age to elucidate developmental mechanisms. An obvious difference is the gestation period, which is 9 months in human and 3 weeks in mice[25]. Sarkar and colleagues (2008) found that the secondary transition, which is characterized in mice by a wave of $Ngn3^+$ cells differentiating into islets and by an increase in β-cells during a short period of time, occurs in human throughout the observation period between 9 and 23 weeks of gestation[24]. This has also been confirmed in other studies[27, 28]. The morphology of the islets differs between mouse and man, where β- and α-cells in human islets are scattered around while in mouse, β- cells are centrally located and surrounded by the other endocrine cells [21, 22]. Rodent β-cells have a greater replication capacity compared with human, as described more in detail below in paragraph 1.5. We and others hypothesize that the use of human pluripotent stem cells may elucidate developmental steps and mechanistic insights in human pancreas.

1.3 **Histology of the pancreas**

The mammalian pancreas is a compound gland composed of endocrine and exocrine tissues. The endocrine tissue consists of five endocrine cell types: β-cells which produce insulin and an insulin antagonist called amylin, α-cells, which produce glucagon, δ-cells which produce somatostatin, ε-cells which produce ghrelin and γ-cells which produce pancreatic polypeptide (PP). These endocrine cells are contained in islets of Langerhans, which comprise 1-2% of the cellular mass of the adult pancreas[29]. Endocrine cells making glucagon, somatostatin, amylin and peptide YY are present not only in the pancreas, but also elsewhere in the gut[16]. The islets of Langerhans are scattered throughout the pancreas and form spheroidal clusters embedded in the exocrine tissue. They have a highly developed blood supply, whereby up to a few arteries enter to the center of the islet, from which they branch into glomerular structures, where collecting venules eventually drain into the portal vein[30]. There is also extensive lymphatic drainage, and a rich sympathetic and parasympathetic nerve supply. Smooth muscle is found in the sphincter of the pancreatic duct, the sphincter of the bile duct, and the hepatopancreatic sphincter. The fibroblastic, lymphatic and smooth muscle components of the pancreas are presumed to arise from the abundant mesenchyme enveloping the embryonic buds[16]. Of the endocrine cells, ~60-80% are insulin-producing β-cells, 15-20% are glucagon-producing α-cells, 5-10% are somatostatin-producing δ-cells and <2% are pancreatic-polypeptide-producing cells. Insulin is released from β-cells in response to increased levels of blood-sugar after food intake; this is a signal for the target tissues (liver, muscle and fat) to take up glucose. In addition, insulin inhibits glucose production in the liver. In contrast, glucagon secretion is stimulated at low blood-sugar levels. Glucagon promotes glycogenolysis and gluconeogenesis as the level of glucose in the blood decreases. Somatostatin and pancreatic polypeptide exert inhibitory effects on both pancreatic endocrine and exocrine secretion[8]. All islet cell types also express, in addition to their specific hormones, a number of gene products characteristic of neuroendocriene cells, such as neuron-specific enolase and the homeodomain-LIM protein islet1 (Isl1)[16]. The exocrine tissue is organized into acini, which synthesize digestive enzymes, such as proteases, lipases and nucleases, and ducts, which secrete a bicarbonate fluid that flushes the acinar secretions to the intestine[29]. These digestive enzymes are secreted as inactive precursors and become activated after they enter the duodenum[16].

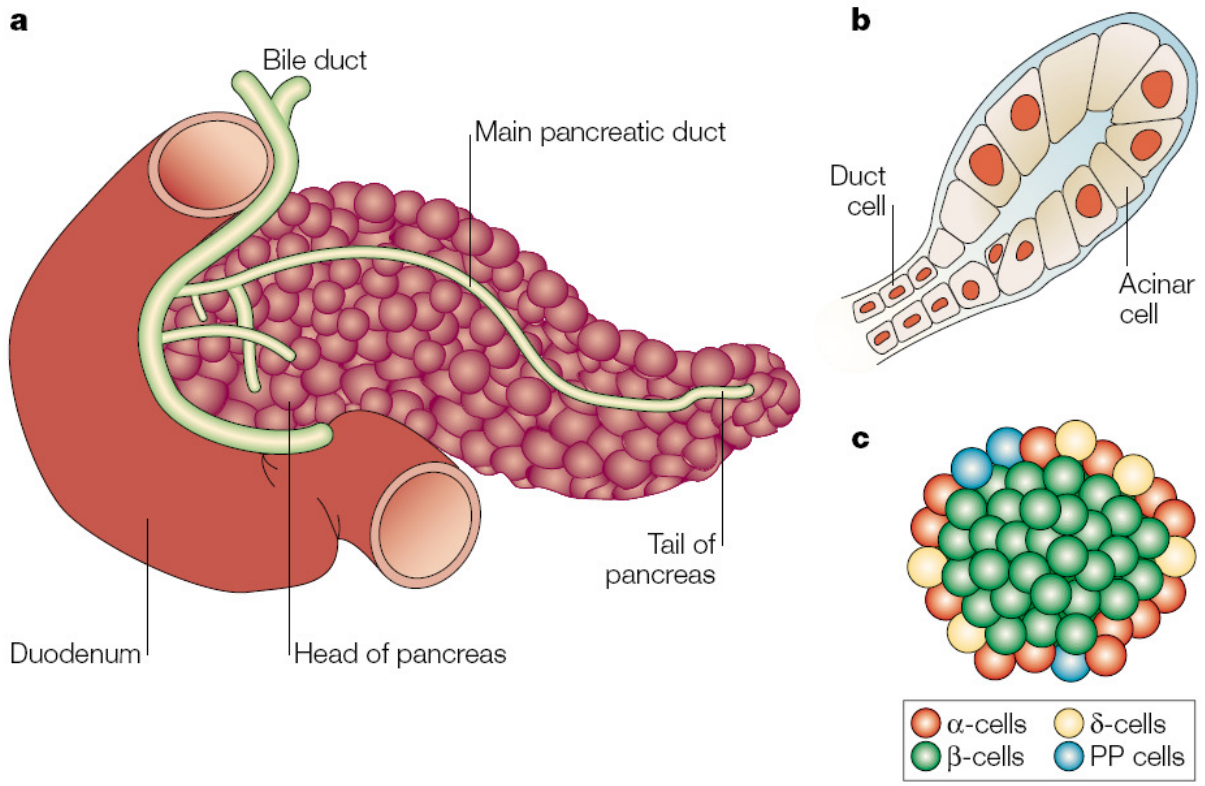


Figure 3. Histology of the pancreas. (a) The pancreas can be found near the duodenum, which is the most anterior part of the small intestine. By making a cross-section of the pancreas, two tissue types can be found: (b) The exocrine pancreas is organized in acini which take care of the secretion of digestive enzymes and ducts which are responsible for flushing the acinar secretions towards the intestine. (c) The endocrine part which is composed of islets of Langerhans. These islets consists of five endocrine cell types: β-cells which produce insulin and an insulin antagonist called amylin, α-cells, which produce glucagon, δ-cells which produce somatostatin, ε-cells which produce ghrelin (not shown) and γ-cells which produce pancreatic polypeptide (PP) (taken from Edlund 2002)

1.4 **Pancreatic transcription factors**

The sequential activation of transcription factor (TF) pathways is essential for the development of the pancreas. It seems that some TFs have dual function in both early cellular commitment as well as maintaining a mature phenotype. The TF cascade depicted in Figure 4 is a simplified schematic overview of the different TFs involved in pancreatic specification, starting with *Pdx1*.

Pdx1, a member of the ParaHox group of homeodomain transcription factors, is expressed in endoderm and has an important regulatory property in pancreatic development[31]. During organogenesis, *Pdx1* is widely expressed in all cells differentiating towards exocrine and endocrine components of the pancreas. As the exocrine pancreas appears and the islets begin to form into the hormone-producing cells, *Pdx1* expression shifts to the endocrine compartment primarily to β- and some subpopulations of δ-cells, PP-cells and a few α-cells. *Pdx1* (previously also named *Idx1* and *ipf1*) is typical example of a TF with multiple functions, as it acquires novel functional properties depending on the gestational age of the embryo. Its action evolves from a growth and differentiation factor during embryogenesis, to the regulatory TF for several proteins of the islets of Langerhans in adults, like *Ins*, *Sst*, *Glut2* (*Glucose transporter 2*), *islet amyloid polypeptide* and *glucokinase*. *Pdx1* is also capable of regaining its initial functions when it becomes required for the expansion of islet-cell mass during adulthood[32].

Another TF which is expressed very early is *Ptf1a*. This gene is important for committing foregut endoderm towards a pancreatic fate. The Wright group (2002) demonstrated that aside from directing pancreatic endoderm cells towards an exocrine fate, it is also a mark for multipotent progenitors of the pancreas. Although endocrine cells could still arise upon *Ptf1a* deletion, a small pool of endocrine cells are generated from progenitors which do not express *Ptf1a*[33].

Seymour and colleagues (2007) suggested that *Sox9* marks and maintains undifferentiated pluripotent pancreatic progenitors. The expression of *Sox9* does not co-localize with the expression of *Ngn3*, which would mean that *Sox9* mRNA expression is restricted from endocrine committed cells[34].

Ngn3 expression appears to be involved in the development of all endocrine cell types. This suggests its importance in the genesis of the islet cell precursor lineage. Pancreatic endocrine cells develop from $Ngn3^{+}$/$Pdx1^{+}$ precursor cells[35]. Apelqvist *et al.* (1999) proved this by creating mice wherein the *Pdx1* promoter induced expression of *Ngn3*, which resulted in a massive

differentiation of pancreatic progenitor cells into endocrine cells. This acceleration and premature differentiation of endocrine cells lead to a shortage of cells that can proliferate, branch and differentiate into exocrine cells[36]. Gu and colleagues (2002) demonstrated unequivocal by lineage tracing studies that *Ngn3*-expressing cells are the islet progenitors of the pancreas[37]. *Ngn3* is also important for the expression of *NeuroD1*, as *Ngn3*-deficient mice do not express NeuroD1. This suggests that *NeuroD1* is located downstream of *Ngn3*. In contrast to *Ngn3, NeuroD1* is still expressed in mature islets[38].

Cells reaching the Ngn3 state have to make a decision of which endocrine cell type to become. One of these faith decisions is regulated by *Pax4*, a gene part of the *Pax* (*Paired box*) gene family and expressed in early pancreas. Sosa-Pineda *et al.* (1997) inactivated the *Pax4* gene by homologous recombination; this resulted in the absence of mature β- and δ-cells, with a considerably higher number of α-cells. Their findings suggests that *Pax4* is not required for generation of the earliest α and β precursors, but assign the gene an early role in the initial processes of endocrine differentiation[39]. Whereas *Pax4* seem to have a key role in β-cell development, Collombat and colleagues (2003) were able to show equal importance of the *Arx* gene for α-cells as *Pax4* for β-cells. *Arx*-deficient mice completely lack glucagon-producing cells. Interestingly, *Pax4* and *Arx* appear to have opposing actions on the specification of mature endocrine cells, which are possible thanks to an inhibitory cross-regulatory network[40].

Nkx2.2 and *Nkx6.1*, members of the NK homeodomain gene family, also play an important role in endocrine specification. Sussel *et al.* (1998) showed that disruption of the *Nkx2.2* gene leads to the accumulation of incompletely differentiated β-cells. These cells express some β-cell markers, but not insulin, indicating an arrest at a partially differentiated state. They also revealed that *Nkx2.2* is involved in the differentiation of α- and PP-cells. However, some α- and PP-expressing cells remain in the mutant islet clusters, however at reduced numbers, suggesting that there may be another regulator that can compensate for the loss of *Nkx2.2*. The δ-cells are not affected by the loss of *Nkx2.2* gene expression. It is likely that the formation of δ cells requires other signals in addition to the absence of *Nkx2.2*[41]. The expression of *Nkx6.1* during embryogenesis and in adults is similar to *Nkx2.2* expression with the exception that *Nkx6.1* is not expressed in non-β islet cells. Sander *et al.* (2000) examined the role of *Nkx6.1* in the hierarchy of transcriptional events that lead to β-cell differentiation. Homozygous mutation of the *Nkx6.1* gene in mice

resulted in a profound inhibition of β-cell formation, thereby preventing the normal expansion of the β-cell population. The phenotype of mice carrying the homozygous double mutation for *Nkx2.2* and *Nkx6.1* has an identical phenotype to mice carrying the *Nkx2.2* homozygous single mutation. This study provided the genetic evidence that *Nkx6.1* lies downstream of *Nkx2.2* in the major pathway of β-cell formation. Sander and colleagues (2000) also showed a decrease in β-cell neogenesis in homozygous single *Nkx6.1* mutant embryos, thereby suggesting that this is due to a defect in the late-stage precursors that have progressed beyond the *Ngn3* stage. This shows that *Ngn3* expression does not depend on *Nkx6.1*[42]. Normal pancreatic organogenesis is initiated when two independent regions, dorsal and ventral of the duodenal portion of the primitive gut become specified for pancreatic development. Not only is an elevation of *Pdx1* expression detected, but also an elevated expression of *Nkx6.1*[15].

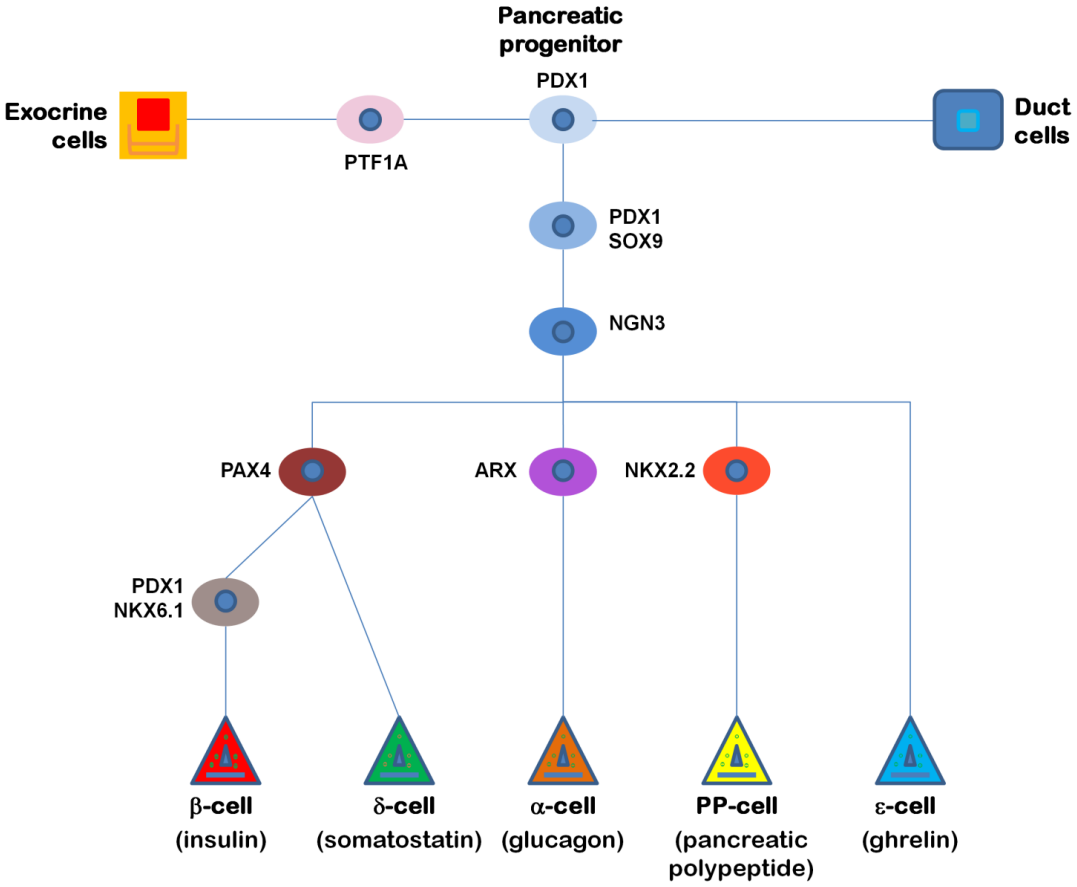


Figure 4 Schematic overview of several transcription factors involved in pancreatic specification on the basis of temporal expression. Circular cells represent intermediate states between the pancreatic progenitor, which express PDX1 and mature cells (indicated with rectangles or triangles) during development. Different transcription factors are represented along with the intermediate states to show their importance for cell specification.

1.5 **Replication and neogenesis of β-cells**

Replication and neogenesis of endodermal cells is far better understood in liver compared to pancreas. Upon a rather mild injury, hepatocytes re-initiate cell cycle and proliferation of mature cells restores the liver mass. However, after a more extensive injury, proliferation of hepatocytes is insufficient to replace the liver mass, and facultative hepatic stem cells, located in the periductal area, can aid in repopulating specific compartments of the liver mass[43, 44]. It has long been unclear whether a similar repair mechanism exists in the pancreas. However, a number of recent studies have suggested that similar mechanisms may be operative in the mouse pancreas. Whether this is also true for the human pancreas remains less clear.

a. Replication of pre-existing β-cells

Dor and colleagues (2004) demonstrated using lineage tracing that pre-existing β–cells have the ability to replicate. They generated a rat-insulin-promoter tamoxifen-dependent Cre recombinase transgenic mouse. When crossed with a floxed human placental alkaline phosphatase (HPAP) mouse, administration of tamoxifen leads to the Cre-mediated removal of a stop sequence which allows the expression of the *HPAP* gene. Therefore, all pre-existing insulin positive cells as well as their progeny will be $HPAP^+$. They demonstrated that new islets generated in mice were derived from pre-existing β–cells, as no dilution of labelled cells could be detected. This however did not exclude the possibility that a small and rare population of stem or progenitor cells is present in the adult pancreas that contributes to the β–cell pool. They also performed a partial pancreatectomy, and found that the majority of β–cells were $HPAP^+$, suggesting that as in the liver, injury to the mouse pancreas results in replication of mature β–cells to reconstitute the pool of β–cells[45]. However, as the injury was relatively modest, these studies could not exclude that β–cells would de-differentiate to a stem/progenitor fate and then proliferate and give rise to mature β–cell progeny.

Subsequently the same group demonstrated that replication of β–cells and not de-differentiation to a more immature progenitor can support regeneration of β–cells in murine pancreata[46]. For these studies they used mice wherein the diphteria toxin receptor is introduced under the

regulation of the insulin promoter. In these mice β–cells can be ablated by administration of doxycycline, which causes activation of the diphtheria toxin receptor in β–cells specifically, and hence provides control over the onset and the termination of β–cell kill. When four-week-old mice were treated with doxycycline for 1 week, 70-80% of β–cells died, and mice became hyperglycaemic. However, after 8 months mice became normoglycemic. To define whether this was due to replication of β–cells, they used the tamoxifen-Cre model described by Dor *et al*. 2004, in combination with BrdU labelling[45]. They found that the majority of $HPAP^+$ cells were $BrdU^+$ and the majority of $BrdU^+$ cells were $HPAP^+$. This demonstrated that the majority of new β–cells are derived from $HPAP^+$ cells that proliferated during the period of BrdU administration, and that few if any other cells contribute to the regenerated β–cell pool. There is also evidence that under steady state conditions β–cells replicate slowly, which increases during pregnancy, following 50% partial pancreatectomy or by adding Exendin-4, a glucagon-like-peptide (GLP)-1 agonist[47].

The frequency of β–cell replication in humans is difficult to investigate, as lineage tracing is not possible. Different approaches have been used to address this question. Gershengorn and colleagues (2004) demonstrated that human islet-derived precursor cells dedifferentiate into fibroblast-like cells, and can be redifferentiated into hormone-expressing islet-like cells through a reversible epithelial-to-mesenchymal transition (EMT)[48]. This is in line with the "self-duplicating β-cell"-theory advanced by *Dor et al.* (2004), which now is shown to make use of the EMT. The second approach used abdominal computer tomographies in 135 children between 4 weeks and 20 years. Morphometric analyses of the tail of the pancreas showed that the β–cell mass expand from birth to adulthood by an increase in islet size rather than increase in the number of islets[49]. Staining pancreata with an anti-Ki67 antibody demonstrated that replicating β–cells peak during infancy. Reers *et al.* (2009) also showed that replication (one positive cell in five islets) is a rare event that this is age-dependent, and that reduced *PDX1* expression was associated with impaired islet turnover in older subjects[50]. Butler and colleagues (2010) looked at islets of pregnant women and found that an increase in β-cell number was due to the emergence of newly formed small islets rather than to an increase in β-cell number of pre-existing islets[51]. The latter observation is not in concordance with studies performed in mice[52], and points out to a

difference in adaptation between human and rodents. Therefore, more investigation in human subjects will be needed to confirm previous results.

b. Neogenesis of β-cells

Although the studies above demonstrate that β–cell proliferation occurs in pancreata, they do not exclude that as in the liver, following extensive damage; (facultative) progenitors are responsible for β–cell neogenesis. As progenitors for endocrine cells in the pancreas express the transcription factor *Ngn3*, a number of investigators have assessed whether $Ngn3^+$ cells can be found in adult pancreas. Forced ectopic expression of *Ngn3* leads to a massive differentiation of pancreatic progenitor cells into endocrine cells. Moreover, $Ngn3^{-/-}$ mice do not develop endocrine hormone producing cells, and die, consequently, at birth [36]. Upon maturation of islet cells, Ngn3 is no longer expressed[38].

Although $Ngn3^+$ cells could not be detected in pancreata following a 50% partial pancreatectomy[53], $Ngn3^+$ cells could be selected from adult pancreata after ductal ligation[54]. Using a lineage tracing technique that permanently labels glucagon- and insulin-positive cells, Xu *et al.* (2008) could rule out the possibility that islet cells de-differentiated to generate $Ngn3^+$ progenitor cells that then re-differentiate to hormone positive cells. The $Ngn3^+$ cells could be found lining the duct of the adult mouse pancreas, and may reflect the pancreas equivalent of the hepatic progenitors that are also found in the periductal region[43]. Collombat *et al.* (2009) demonstrated that overexpressing *Pax4* in glucagon-expressing cells, leads to refating to β–cells, but this occurred via an $Ngn3^+$ intermediate state, suggesting de- and re-differentiation[55]. The previous studies strongly suggest the existence of a pancreatic endocrine progenitor cells in adult tissue in mice. It is not clear where these cells originate from. Some reports have suggested that in human pancreatic the ductal compartment tissue is the source of newly generated β-cells[56, 57].

1.6 **Alternative cell sources for β-cell therapy**

a. Reprogramming towards β-cells

The reprogramming of different cell types towards β-cells has been successful in mice. Therefore most of the reports mentioned in this section are from rodent studies. Whether, the findings also hold true for human cells remains to be determined, as there is evidence for species specific differences in ontogeny and regeneration of endocrine pancreas.

Liver cells

Refating endodermal cells to β–cells can be achieved by transgenic overexpression of pancreas/endocrine-specific TFs. One of the first reports demonstrated that *in vivo* introduction of the *Pdx1* transgene into hepatocytes can activate β–cell features[58]. Using a first-generation andenoviral vector *Pdx1* was ectopically expressed in the liver of 14-week old BALB/c and C57BL/6 mice. This induced expression of *Ins-2*, but not *Ins-1*. When *Pdx1* as well as a rat *Ins-1* promoter (RIP) followed by the human *INS* cDNA, were co-expressed, human *INS* could be detected. This suggested that the promoter of Ins1 in mouse hepatocytes might be methylated or silenced in another manner. Immunoreactive INS could also be found in extracts derived from hepatic tissue, even if only at 1% of immunoreactive insulin in pancreas extracts. To assess if hepatic insulin production could reverse hyperglycemia, mice were first made diabetic and then treated with the *Pdx1* encoding adenovector. Although glycemia levels decreased on day 2, it is unclear whether this was due to insulin or by *glucokinase*, which was also induced following overexpression of *Pdx1*. In addition, which cells were transduced and partially refated in the liver was unclear. *In vitro* data showed that primary cultures of hepatocytes could not be transdifferentiated, suggesting that perhaps a progenitor population in the hepatocytes was targeted *in vivo*.

As the first generation adenoviral vectors are hepatotoxic, Kojima et *al.* (2000) used helper-dependent adeno (HDAD)-viral vectors, which do not have the same toxic effects as adenoviral vectors to trans-differentiate hepatocytes to β–cells[59]. They over-expressed *Pdx1* using HDAD-

viral vectors in diabetic mice. An effect on glycemia was seen for only one week. The authors speculated that is due to the fact that *Pdx1* also induced the expression of several pancreatic exocrine enzymes that lead to the destruction of the newly generated β-cells. Increasing the dose of viral vector did not prolong the effect on blood glucose levels, but caused toxicity instead, and animals died 4 weeks after infusion of the *Pdx1* containing HDAD-viral vectors, but not with empty vector. As the mice in the study by Ferber *et al.* 2000 were not analyzed for more than 8 days, this hepatoxic effect of *Pdx1* due to the use of the first-generation adenovector was not seen in their study[58].

In a subsequent study the effect of overexpression of *NeuroD1* alone or combined with *betacellulin* in the liver of diabetic mice was tested. This resulted in normalization of blood glucose levels within 3 weeks, which was maintained for up to 120 days. Several β–cell specific transcripts, including *Ins-1*, *Ins-2*, *Pcsk1, Psck2, Kcnj11 and Abcc8* could be detected in the transduced liver. Also, the pancreas-specific *glucokinase* mRNA was detectable. In addition, TFs downstream from *NeuroD1* as well as endogenous *NeuroD1* transcripts could be detected. The liver contained structures with the morphology of islets with 30-50 insulin-positive cells. The islets contained also cells with ultrastructural features of β–cells, i.e. secretory granules. The number of newly formed islets was comparable to the number of islets transplanted in humans[60].

Another study tested the effect of forced expression of Ngn3 in hepatocytes. They transduced the liver with a HDAD viral vector encoding for the *Ngn3* and *betacellulin* (*Btc*) gene. Using lineage tracing, they demonstrated that initially cells could be identified that co-express Alb and Ins which was followed in a second phase by transduction of periportal oval cells[61], which were responsible for β-cell neogenesis. This resulted in the reversal of DM, as well as in normalization of glucose secretion and reversed ketonemia. Further, the transcriptome of the neo-islets generated in the liver was highly similar to that of primary pancreas derived islets. The authors used the term trans-determination instead of trans-differentiation to indicate that the fate switch was caused by altered fating of endoderm progenitors, and not from mature hepatic cells[60].

Similar studies have been performed using human hepatic cells *in vitro*. Zalzman and colleagues (2005) immortalized human liver derived epithelial cells, which are capable of generating biliary cells and hepatocytes. They subsequently transduced this cell line with the rat *Pdx1* gene, and

demonstrated that the transduced cells could secrete INS upon glucose stimulation, and expressed transcripts for *NGN3, NEUROD1* and *NKX6.1*. However, the cells continued to express hepatocyte specific gens such as *DPPIV* and *γ-glutamyl transpeptidase*, and stained positive for GLYCOGEN. Nevertheless, the cells could restore euglycemia in diabetic mice 2 weeks after transplantation[62]. An improved protocol was published by the same group, which increased the expression of several genes important in the development of β–cells including *NEUROD1*, *NKX2.2*, *GCK* and *PC 1/3* and established more quickly stable euglycemia[62]. As transduction of *Pdx1* in murine or rat hepatocytes can refate them to β-cells[58], this also appears to be possible in immortalized human hepatic progenitor cells[62, 63].

In another approach, human adult and fetal hepatocytes were transduced with a bi-functional adenoviral construct containing a rat insulin promoter-driven green fluorescent protein (GFP) and a *PDX1* gene under the control of a cytomegalovirus (CMV) promoter. In ± 10-25% of the adult human hepatocytes the insulin promoter became activated, which was not significantly different from what was seen in fetal hepatocytes. The transdifferentiated adult cells produced insulin following glucose administration, and restored normoglycemia *in vivo*. This led to the conclusion that the fetal or adult origin of the hepatocytes does not have a substantial effect on trans-differentiation[64].

Pancreatic endocrine cells

Zhou *et al.* 2008 evaluated several TFs that may redirect the phenotype of exocrine pancreas cells to insulin producing β-cells. They identified 9 TFs that when mutated cause developmental β-cell defects. As adenoviral vectors transduce endocrine cells very poorly, they were sure to transduce mainly exocrine cells. The transduction of 9 factors together with nuclear GFP encoded by adenoviral vectors caused the appearance of GFP positive transduced cells that co-express insulin[65]. They narrowed down the mix of TFs to *Ngn3*, *Pdx1* and *MafA* as being necessary to reprogram exocrine cells to insulin-positive cells. The insulin-positive cells did no longer express exocrine marker genes (such as *amylase* or *Ptf1a*), mesenchymal markers (such as *nestin* and *vimentin*) or the neuronal marker *Tuji*. They also did not express any of the other pancreatic endocrine hormones. To definitively prove that an exocrine pancreatic cell was trans-

differentiated, a genetically modified mouse line (*Cpa1CreER^{T2}*) was crossed with the *R26R* reporter line, to label mature exocrine cells with β-galactosidase *in vivo*[66]. Most of the β-galactosidase$^+$ cells became insulin$^+$ after transduction with *Ngn3*, *Pdx1* and *MafA*. The trans-differentiated cells could revert hyperglycemia *in vivo.* To exclude de-differentiation, which is characterized by a rapid division and expression of *Sox9* and *Hnf6*, they looked at BrdU incorporation of the refated cells. Only 3.2% were BrdU$^+$, whereas no *Sox9* and *Hnf6* expression could be detected. This led them to conclude that the newly formed β-cells were likely to be generated through trans-differentiation and not through de-differentiation and subsequent re-differentiation of exocrine pancreatic cells.

b. Differentiation of other adult non-β-cell sources

Bone marrow cells

There has also been interest in testing whether stem cells from bone marrow (BM) might be able to generate hormone producing pancreatic cells. One study demonstrated that BM cells might be capable of generating β–cells[67]. Following intravenous infusion of INS-GFP male donor BM cells, these cells could be found back in the pancreas of the female recipients. The group also used BM cells from INS-CRE male mice in female ROSA-stop-loxP-eGFP mice, to exclude fusion events, since no GFP-expressing cells could be identified. They suggested that BM cells could trans-differentiate into hormone producing β–cells. However, others[68] have been unable to reproduce the studies by Ianus et al. (2003) using the same model and including others like partial pancreatectomy and streptozotocin-induced destruction of β–cells.

A few studies tried to determine whether human BM cells would be able to trans-differentiate into β–cells. Butler and colleagues (2007) examined 31 human pancreata obtained from patients who underwent hematopoietic stem cell transplantation from a donor of the opposite sex. Using in situ hybridization they examined the presence of the Y chromosome in β-cells, but were unable to identify male cells that were pancreatic β–cells in nondiabetic humans[69].

Mesenchymal stem cells

Mesenchymal stem/stromal cells (MSC) were first described in the 1970s by Friedenstein *et al.* (1974) as fibroblast colony forming cells[70]. In the 1990s, the cells were renamed mesenchymal stem/stromal cells by Caplan and others[71-73] . MSCs are multipotent progenitor cells that can give rise to osteoblasts, chondrocytes, adipocytes and smooth muscle cells. MSCs can be harvested from among other tissues, BM, adipose tissue, fetal lung, Wharton's jelly, amniotic fluid[72, 73]. It is unclear whether MSCs can generate β–cells. Lee and colleagues (2006) injected human MSCs intra-cardially and found an amelioration of glucose levels in diabetic mice, although no circulating human insulin levels were detected. In the mouse pancreata, they found an increased immunoreactivity for mouse insulin and an increase in islet number compared to untreated diabetic mice, suggesting that human MSCs can increase insulin secretion of mouse β-cells[74]. Sordi *et al.* (2008) isolated MCSs from pancreas and infused them into diabetic mice. An improvement in the control of glycemia was observed due to the improved vascularization induced by the MSCs[75]. Some studies do suggested that MSCs can generate β-cells when injected systemically into the tail vein of diabetic mice[76]. Therefore it is still controversial whether MSCs have the capacity to give rise to lineages other than mesoderm.

Human pluripotent stem cells (hPSCs)

Embryonic stem cells (ESCs) are pluripotent as they have the ability to differentiate in all cell types of the mouse or human, including germ cells, and have indefinite self-renewing ability. Although murine ESCs were first isolated in 1980, it was only in 1998 that human ESCs were isolated from embryos generated from *in vitro* fertilization[77]. Human ESCs express cell surface markers like TRA-1-60, TRA-1-81, stage-specific embryonic antigen (SSEA)-3, SSEA4 and alkaline phosphatase. The use of human ESCs is however ethically encumbered, as it requires the use of human embryos. In 2006 and in 2007, the team of S Yamanaka identified a method to de-differentiate any adult cells from mouse or human, or other species to induced pluripotent stem cells (iPSCs) by overexpression of *OCT4*, *SOX2*, *KLF4* and *cMYC*[78]. iPSCs can be derived using integrating vectors such as lentiviral and retroviral vectors; transposons; non-integrating vectors such as adenoviral vectors and Sendai virus; introduction of the TF proteins, modified mRNAs; as well as microRNAs[79]. One main question remains: "How identical are iPSCs compared with

ESCs?". For a more detailed overview concerning this topic, see the review from Robinton and Daley[79]. Mayshar *et al.* (2010) showed that the amplification of chromosome 12 is present in both cell types[80]. This was confirmed by Taapken and colleagues (2011) who also showed that the observed genetic aberrations are independent from cell type, passage number, cell support and reprogramming procedure using a large-scale study of several hundred cell lines[81]. Although there is no difference in the incidence of genetic abnormalities, some reports find different chromosomal alterations in hESCs vs. hiPSCs. A gain of chromosome 1q (1q25-1q41) or trisomy of chromosome 17 are commonly seen in hESCs but not in hiPSCs[80, 81]. There is also an instability effect observed in the epigenome of hPSCs specifically where it concerns the methylation pattern of a subset of imprinted genes as well as X-chromosome inactivation[82]. Guenther and colleagues (2010) looked at histone modifications patterns in hPSCs and observed no differences in the pattern of H3K27me3 and H3K4me3 marks between hESCs and hiPSCs[83]. Also a study performed with mouse pluripotent stem cells reported negligible immunogenicity for both cell types[84], which is important for cell-based therapy. Overall, most studies conclude that hESC and hiPSCs are generally comparable cell lines. Specific attention should be given regards to the cell source from which iPSC are generated, because several studies showed varying reprogramming susceptibility amongst different cell types[85-87]. Daley's group (2010) analyzed the influence of cell type origin and came to the same conclusion as Hochedlinger's group (2010) that this phenomenon can be attributed to the epigenetic memory of the donor tissue, which has to be fully erased to establish the ground state of pluripotency. If not, residual epigenetic marks may influence the differentiation potential of iPSC-progeny[88, 89], although Pole *et al.* (2010) demonstrated that long-term passage of iPSCs from different cell sources erases many of the epigenetic differences present during early passage[89]. Another example is that upon reprogramming of β-cells, the iPSC still retain an open chromatin structure as well as a unique methylation signature compared to other hPSCs. The β-cell derived iPSCs used in this study had a greater tendency to differentiate towards insulin-expressing cells compared to non-β-cell iPSCs[90].

To induce pancreatic cells from hPSCs, it is important to recapitulate *in vivo* development starting from the blastocyst stage, through the primitive streak, passing through definitive endoderm, gut tube endoderm (GT), anterior foregut (AF), pancreatic endoderm and finally

towards endocrine hormone-producing cells. It is generally acknowledged that the use of Activin-A induces definitive endoderm in ESCs[91] and can be improved with Wnt3A[92]. Sonic Hedgehog (Shh) which is known to repress pancreatic endocrine differentiation[93] must be inhibited, to allow the further development into GT and AF. Retinoic acid (RA) and NOGGIN, which blocks bone morphogenetic protein (BMP) signaling, are crucial to generate pancreatic endoderm[94]. D'Amour *et al.* (2006) have demonstrated that it is possible to generate pancreatic hormone-expressing endocrine cells from hESCs, using signals described above that regulate pancreas development *in vivo*. hESC progeny expressed pancreatic hormones like insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin, but the differentiated cells were only minimal glucose-responsive[92]. In addition, many of the *in vitro* generated progeny consisted of poly-hormonal cells. They subsequently adapted the protocol and grafted hESC derived endocrine progenitors, expressing mainly *PDX1* and *NKX6.1*, *in vivo* to allow further maturation. Glucose-responsive cells were formed *in vivo* 30 days after grafting the ESC progeny in euglycemic mice. To demonstrate that the graft was capable of producing insulin, they eliminated the β–cells of the mice after transplantation using streptozotocin, which does not affect human β–cells and demonstrated persistent euglycemia for several months[95]. However, this was only possible with one hESC line, and other groups, using similar endodermal committed cells could only partially reproduce these findings, showing only limited endocrine cell development after a much longer time period than described before[96].

Reports that hiPSC can be induced to differentiate to endocrine pancreatic cells have not yet been published. Alipio and colleagues (2010) succeeded in reversing the hyperglycemia of diabetic mouse models using murine iPSC-derived pancreatic β–like cells. They made use of two different mouse models which represent both type 1 and type 2 DM to demonstrate that iPSC derived progeny is able to reduce insulin resistance and reverse glucose toxicity[97].

Thus, although it appears possible to induce differentiation of ESC towards definitive endoderm, and also towards pancreatic endoderm, full maturation to terminally differentiated β–cells remains difficult to achieve. Some groups have therefore started high-throughput compound screens to identify novel molecules that might aid in the different commitment steps. Using this approach, the Melton group identified molecules that either improve commitment to definitive endoderm (IDE1 and IDE2), or commitment to pancreatic endoderm (indolactam V (ILV)[98, 99].

Even if fully mature β–cells could be generated, the possibility that the final differentiated product contains remaining pluripotent cells that can form teratomas still needs to be overcome before ESC progeny can be used clinically. One method to eliminate this risk is to positively isolate endocrine progenitors or more mature hormonal cells using antibodies against cell surface antigens. To achieve this, reporter cell lines would aid enormously. For instance a SOX17-GFP reporter cell line was created[100] using bacterial artificial chromosome (BAC) recombineering. $SOX17^+$ cells were isolated and used for the identification of cell surface antigens for endoderm progenitors. Also large fluorescence activated cell sorting (FACS)-based screens can be performed and has led to the identification of the $CD149^+$ surface marker which can be used to enrich for $PDX1^+/NKX6.1^+/PTF1A^+$ cells. $CD149^+$ cells are multipotent pancreatic progenitors that can differentiate into all pancreatic lineages[101].

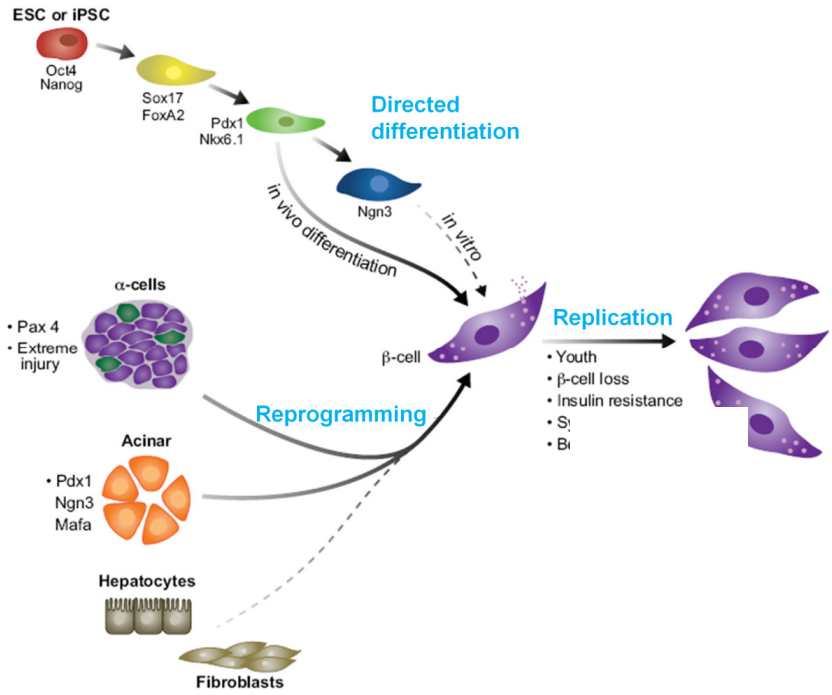


Figure 5 Overview of different strategies for the generation of β-cells. (a) It is possible to direct pluripotent stem cells (red) through the stages of pancreatic differentiation with growth factors by mimicking normal development. Unfortunately, functional β-cells can only be generated through an in vivo transplantation step, but deriving a bona fide β-cell fully in vitro (dashed line) is a major goal. A subset of important genes expressed at each stage is listed. (b) Reprogramming of terminally differentiated cell types, such as acinar or α-cells, can be used to generate β-cells in vivo, using the strategies discussed. Reprogramming other mature celltypes, such as hepatocytes, fibroblasts in vitro into β-cells (dashed line) remains to be achieved. (c) Inducing the replication of existing β-cells is the most interesting strategy for generating new endogenous β-cells. Replication may be recapitulated in vitro or induced in vivo with new small molecules or proteins. (adapted from Pagliua and Melton, 2013[102])

1.7 **Genome editing of PSCs**

The extensive differentiation and regenerative potential of PSCs makes them a very interesting tool for studying human developmental, and perhaps even for therapeutic approaches. Although progress is being made in differentiating PSCs to mature cell types, in general PSC progeny remains impure and mixed populations after sequential differentiation of the PSCs towards cell types of multiple organs like liver, pancreas, intestine and lung. Therefore, the prospective isolation of specific intermediary and mature cell types would be helpful in dissecting the cultures, as well as to bypass the issue of transplanting undifferentiated PSCs that can give rise to teratomas[77, 103]. One method for selecting specific cell types is based on antibodies against specific cell-surface markers for each cell type of interest. Unfortunately, a specific cell surface antigen is not available for many intermediary progenitors and mature cells in for instance the pancreas.

Therefore creation of transgenic reporter cell lines would aid enormously to elucidate the molecular signature of hPSCs-derived progenitors and mature cells[100, 104]. The ability to genetically manipulate mouse ESCs has revolutionized the biomedical and biological sciences. Applying this technology to human PSCs, i.e. generation of transgenic or knock-in reporter cell lines that target specific steps during PSC differentiation, would be interesting for many biological studies and transplantation approaches[105-107]. This genome editing in PSCs can be achieved by many ways including amongst others, homologous recombination (HR) using viral vectors, bacterial artificial chromosome, zinc finger nucleases (ZFNs), transcription activator-like effector nuclease (TALENs)[107-109]. A more recent genome editing technique has been developed which is termed: clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (CAS) system. Upon complementarity between an RNA and DNA sequence, the Cas9 protein will be directed towards the site to allow cleavage of the DNA[110]. The CRIPSER/CAS9 system is more efficient than TALENs, even if it is not yet clear how many off target cuts will occur[111]. An overview of the efficiency of the different genome editing techniques was taken from Cheng *et al.* 2012 and is shown in Table 2. Previous reports using genetically modified hPSCs already showed the power of this application for the prospective isolation of certain cell types for developmental studies. For example, Wang and colleagues (2011) were successful in isolating *SOX17*-expressing cells from differentiating

hESCs. They showed that these cells could further develop into liver, pancreas and intestinal epithelium, rendering it an interesting tool to analyze developing endoderm. In addition, they could indentify surface markers for the *SOX17*-expressing endoderm progenitor[100]. Another group also made use of a knock-in, this time for the *NKX2.5* gene. When directing this reporter cell line towards a cardiac fate, they could identify *NKX2.5*-expressing cells as human cardiac progenitors and cardiomyocytes. By doing so, they also identified specific surface markers for these cells[112]. Other groups reported the use of a knock-in for the *INS* gene in hPSCs. Unfortunately, the cells were not glucose responsive *in vitro*[113, 114] and upon transplantation in mice, they only differentiated into *GCG*-expressing cells *in vivo*[114]. The outcome of these studies was rather unexpected, but does reflect the importance of the choice of the targeted gene for lineage tracing purposes. It should be noted that the INS positive cells selected in the study by Basford *et al.* (2012), mostly express INS in combination with other hormones, suggesting that the INS-expressing population still contained immature endocrine cells. The authors hypothesize that the maturation into *GCG*-expressing cells could be due to lack of a factor(s) required for the conversion of pancreatic endodermal cells into β-cells. A more recently published study from the Sander's group (2013) has shed more light on this observation. They demonstrated that terminal differentiation into endocrine cells is not possible *in vitro*, due to aberrant histone modification marks. They prove this by comparing the epigenetic profile of *in vivo* matured with *in vitro* matured endocrine cells[115].

Table 2 Overview of different genome editing techniques (taken from Cheng *et al.* 2012). *CRISPRs was not included in the overview, therefore direct comparison is difficult.

	Viral vectors	BAC	ZFN	TALEN	*CRISPRs
Efficiency	1-78%	10%	0.3-48%	11.8-75%	Higher than TALEN
Accuracy	Random	Specific	Specific	Specific	Specific
Positional effect	High	Low	Low	Low	Low

One main hurdle for the targeting of hPSCs is that it is significantly more difficult to clonally select hPSCs, because they undergo apoptosis much more quickly upon dissociation into single cells compared with mPSCs, and hence need to be passaged preferably as cell clumps. This problem can be largely overcome by the use of neurotrophins or ROCK inhibitor Y-27632 that greatly increases the cloning efficiency of hESC without affecting their pluripotency and genomic instability. Pyle *et al.* (2006) made use of the receptors of the tropomyosin-related kinase (TRK) to inhibit hPSC apoptosis. They identified three TRK ligands, neurotrophin 3, neurotrophin 4 and brain-derived neurotrophic factor, which support the survival of single hESC through the phophatidylinositol-3-kinase pathway. Without adding these factors the survival rate of dissociated hESC was lower than 1%[116, 117]. Another group in Japan demonstrated that the selective Rho-associated kinase (ROCK) inhibitor, Y-27632, also diminished dissociation-induced apoptosis and increased the efficiency of clonal hESC growth[118].

We choose to generate NGN3eGFP-hESC lines from H1 and H9 using the ZFN technology combined with the use of Y-27632 to allow for single-cell survival. These genetically modified lines are different from most previously reported transgenic lines, because we make use of a knock-in add-on strategy. This approach retains two intact NGN3 alleles, whereas most studies have created lineage-tracing lines by disrupting one allele of the targeted gene. This ZFN technology is based on the use of a nuclease, FokI, which is linked to three or four zinc fingers. Each zinc finger is able to recognize a specific nucleotide triplet. When two ZFN pairs are in close proximity of each other, a double strand break (dsb) can be induced in the DNA through the FokI domain. In this way, the genome will need to repair the dsb through homologous end-joining or through HR using exogenous homologous DNA, which can be used to introduce transgenic expression cassettes[108]. Besides the very efficient targeting of hPSCs using ZFNs, there are also some disadvantages related to this technology, like the off-target effect and the genotoxicity, as well as the high cost to design a proper ZFN pair. Therefore, much effort has been put in the use of TALEN instead, which would not suffer of previous disadvantages[109].

2 AIMS

Human embryonic stem cells (hESCs) are being evaluated as a possible source of β-cells to treat DM type 1. Investigators world-wide have used growth factors and/or small molecules to mimic pancreatic development *in vitro* to generate insulin-producing β-cells from hESCs[92, 95, 98, 99, 119]. Some groups documented that hESC-derived β-cells, like primary islets, can reverse hyperglycemia in mice immediately following grafting of the hESC progeny[120, 121]. Other groups have grafted mixed ESC progeny consisting chiefly of PDX1- and NKX6.1-expressing cells to reverse hyperglycemia in diabetic mice. However, development of normoglycemia required further *in vivo* maturation of the hESC-derived pancreatic endoderm[95, 119]. Kelly *et al.* (2011) selected pancreatic endoderm progenitors with an anti-CD142 antibody, or endocrine cells with an anti-CD200 and -CD318 antibody. CD142 selected cells yielded islet-like clusters, ductal and exocrine tissue after implantation, whereas CD200- and CD318-enriched population gave rise mainly to glucagon (GCG)-positive cells[101]. All these studies relied on the transplantation of mixed or not completely pure cell populations, which in some cases lead to the generation of teratomas[95, 119]. Therefore, it remains unclear which cell is responsible for the generation of human insulin-producing β-cells.

We hypothesized that the isolation and characterization of a specific pancreatic endocrine progenitor population would provide a useful tool for the characterization of mechanisms that underlie generation of β-cells from hESCs *in vitro* and/or *in vivo*, and could serve eventually as a valuable and safe source for cell therapy. In mouse, Ngn3-expressing cells are known to give rise to all hormone-secreting endocrine cell types in the pancreas[36]. Upon maturation of islet cells, Ngn3 is no longer expressed[38]. Therefore NGN3 might be a useful marker for the identification of human pancreatic endocrine progenitors. We here explored this possibility by selecting NGN3-expressing cells from differentiating hESCs *in vitro*. Zinc finger nuclease (ZFN)-mediated homologous recombination (HR) was used to target a reporter-selectable cassette at the C-terminus of *NGN3*, allowing the isolation, purification and characterization of NGN3-expressing cells.

2.1 **Aim 1: Genetically modify hESCs to generate a reporter cell line for *NGN3***

To address whether *NGN3* plays a role in human cells as shown before in mice by Gu *et al.* 2002[37], we decided to genetically modify hESCs with the ZFN technology to generate a lineage tracing tool for *NGN3*. In doing so, we also checked whether the cells were affected by the targeting experiment through differentiations, teratoma formation assays, the detection of pluripotency markers and array-comparative genomic hybridization (array-CGH).

2.2 **Aim 2: Characterization of the prospectively isolated NGN3eGFP$^+$ cells**

Upon differentiation of the NGN3eGFP-hESC lines, we directed the cells towards a pancreatic endoderm fate to induce NGN3$^+$ cells. These cells were enriched using antibiotic selection and sorted out by fluorescence-activated cell sorting (FACS). Further characterization of these cells was performed by quantitative-real time polymerase chain reaction (qRT-PCR) and immunostaining.

2.3 **Aim 3: Assessing the potential of the NGN3eGFP$^+$ cells**

The potential of these NGN3eGFP$^+$ cells was assessed in an *in vitro* differentiation experiment as well as in an *in vivo* transplantation assay. In this way, we could see if these cells have the potential to generate human pancreatic endocrine cells.

4 MATERIALS AND METHODS

4.1 **Cell culture**

The human ESC lines, H9 and H1 were purchased from Wicell Research Institute (Madison, WI). Experiments were performed at the Katholieke Universiteit Leuven with approval from the Medical Ethics Committee, UZ Leuven, Gasthuisberg. The ESCs were cultured on inactivated mouse embryonic fibroblasts (iMEFs) (250,000 cells/well, Globalstem, GSC-6301G) in DMEM/F12 (Life Technologies, 31330095) supplemented with knockout serum replacement (Life Technologies, 10828010), penicillin and streptomycin (Life Technologies, 15140122), glutamine (Life Technologies, 25030024), nonessential amino acids (Life Technologies, 11140035), basic fibroblast growth factor (bFGF, 4ng/ml, Life Technologies, PHG0023) and β-mecaptoethanol (Sigma, M6250). Passaging at 1:3-4 was done using collagenase IV (1mg/ml, Life Technologies, 17104019) for cells maintained on iMEFs. For differentiation purposes, cells were harvested through enzymatic dissociation and plated on 2% matrigel (Becton Dickinson, 354230) coated wells in mTESR medium (Stem Cell Technologies, 05870). After the cells reached 60-70% confluency, differentiation was initiated as depicted in Figure 7a. Pancreatic differentiation medium consists of 55% DMEM with 1000 mg/L D-Glucose (Life Tecnologies, 31885-023), 2% fetal bovine serum (Hyclone, CH30160.03), 0,1% (0.1 mM) β-mercaptoethanol (Gibco, 31350-010), 0.1% Penicillin-Streptomycin liquid (Life Technologies, 15140122), 40% MCDB 201 medium with trace elements, L-glutamine and 30 mM HEPES (Sigma, M6770), ITS+1 (100×) (Sigma, I2521) , 0.1 mM L-Ascorbic acid (Sigma, A8960). Wnt3a (50ng/ml prepared in PBS containing 0.1% bovine serum albumin (BSA) (Sigma, A-8806), R&D, 5036-WN) and Activin A (100ng/ml prepared in PBS containing 0.1% bovine serum albumin (BSA) (Sigma, A-8806), R&D, 338-AC) are used for stage 1. NOGGIN (100ng/ml prepared in PBS containing 0.1% bovine serum albumin (BSA) (Sigma, A-8806), R&D, 6057-NG), anti-SHH (2.5ug/ml prepared in PBS containing 0.1% bovine serum albumin (BSA) (Sigma, A-8806), R&D, MAB4641), nicotinamide (NA) (10mM prepared in MilliQ water, Sigma, N0636) and retinoic acid (RA) (10μM prepared in ethanol (Sigma, E7023), Sigma, R2625) were added between d4 until d16 (stage 2).

The NGN3eGFP$^+$ enriched cells were cultured as clusters: 10,000 cells/well were plated in 96-well low-attachment plates, Hydrocell 96U Microwell (Thermoscientific, Nunc, 174908) with NOGGIN, anti-SHH, RA and NA as described before. To allow cluster formation, plates were spun at 200*g* for 5min. After two days, medium was replaced with RPMI1640 medium (Life Technologies, 11835105) with 0.05w% BSA (Sigma, A-8806), 0.1% Penicillin-Streptomycin liquid (Life Technologies, 15140122) containing NA (10mM prepared in MilliQ water, Sigma, N0636) and insulin-like growth factor I (IGFII) (50ng/ml prepared in PBS containing 0.1% bovine serum albumin (BSA) (Sigma, A-8806), R&D Systems, 292-G2) for an additional 24 days

4.2 **RNA isolation and quantitative real time-polymerase chain reaction (qRT-PCR)**

Total RNA was extracted using the RNAeasy mini kit (Qiagen, 74104) and cDNA was synthesized from 1µg total RNA using Superscript III reverse transcriptase (Life Technologies, 18080-51) both according to manufacturer's protocol. For qPCR: the cDNA underwent 40 rounds of amplification on a Mastercycler® ep Realplex qPCR instrument (Eppendorf) as follows: 40 cycles of a 2-step PCR (95°C for 15 sec, 60°C for 45 sec) after initial denaturation (50°C for 2min, 95°C for 2 min) using specific primers, Platinum SyBRGreen qPCR supermix-UDG (Life Technologies, 11733-046). and 2µl cDNA. For normalization purposes, *PPIA* was used as a housekeeping control and results are shown in deltaCt = Ct (gene of interest) - Ct (*PPIA*). Primers used were synthesized at IDT Technologies. A list of the primers can be found in Table 3.

4.3 **NGN3 gene targeting**

The ZFNs were designed in collaboration with Sigma. The ZFN binding site is shown in Figure 6a. The validation of the ZFNs was performed by Sigma. Human K562 cells were used to verify the specificity of the ZFNs. The NGN3 ZFNs were able to target 8.7% of the cells (see Figure 6a). Also, the Cel-1 assay performed on the NGN3 gene demonstrated sucessful targeting of the *NGN3* allele (see Figure 6c). The different components for the donor construct for NGN3 were cloned in the pCR2.1 plasmid. For the homology arms of NGN3 we used up to 800bp amplicons

surrounding the stop codon, amplified from the genomic DNA of hESCs, see Appendix for more details.

a GGCCACCTTTTCCGCCTGcttgaGCCCAGGCAGTC

b

Kit Component	Test	Specification	Result
ZFN mRNA	ZFN activity in human K562 cells	>1%	8.7%

c

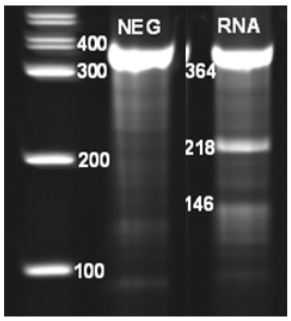


Figure 6. (a) ZFN binding site, in red the cut site is shown. (b) The NGN3 ZFN mRNA is shown to be active in human K562 cells. (c) Cel-1 assay resolved on 10% TBE-PAGE

HESCs cells were dissociated with 0.05% trypsin. For the nucleofection of ZFN mRNA and donor plasmid into hESCs, 2-3 million hESCs per condition were washed twice with PBS (Life Technologies, 20012-019). The nucleofection was performed with the Amaxa nucleofector (Lonza) using nucleofection solution 2 of the hESCs nucleofection kit and with program A34. The nucleofected cells were plated on inactivated DR4 MEFs (Globalstem, Z8030001) containing 10 mM ROCK inhibitor Y-27632 (Sigma, Y0503) and selected with hygromycin (H9:50ug/ml and H1:25ug/ml prepared in MilliQ water, Sigma, H3274) for one to two weeks. Surviving colonies were individually picked and expanded.

4.4 **PCR genotyping**

DNA was extracted from cell pellets using the QiaAmp DNA mini kit (Qiagen, 51306) and 100ng was used to perform the PCR reactions. Junction assay primers (see

a in red) for the targeted integration of our reporter construct with an annealing temperature of 60°C with the Expand High Fidelity PCR system (Roche, 11 732 650 001). Random integration primers (see Figure 7a in blue) were used for detecting random integration of the plasmid with an annealing temperature of 60°C as well, but with the Platinum Taq DNA polymerase High Fidelity (Life Technologies, 11304011).

4.5 **Teratoma formation and analysis**

Three to four wells of HESCs were collected with collagenase IV and were resuspended in 120µl PBS (Life Technologies, 20012-019) and injected with 120µl matrigel (Becton Dickinson, 354230) subcutaneously in the back of severe combined immunodeficient (RAG2γc) mice. Tumors generally developed within 4–8 weeks. Animals were killed before teratoma collection. Teratoma were fixed in 4% paraformaldehyde (overnight) and subsequently embedded in paraffin. After sectioning, presence of cells from three germ layers was assessed following hematoxylin and eosin staining.

4.6 **Southern blot**

Genomic DNA was separated on a 0.7% agarose gel after AvrII (Fermentas, ER1561) restriction digest, transferred to a Zeta-probe membrane (BioRad, 162-0165) and hybridized with probes labeled with digoxigenin using the PCR DIG Probe Synthesis Kit (Roche, 11636090910).

4.7 **Array Comparative Genomic Hybridization (aCGH) and karyotyping**

Genomic DNA isolated from NGN3eGFP-H9 (three different clones, N=2 each clone) and WT hESCs (N=2) all having passage numbers between 40 and 57 using QiaAmp DNA mini kit (Qiagen) were subjected for CNV analysis on 180k Cytosure ISCA v2 arrays (Oxford Gene Technology, Oxford, UK). The karyotype of the NGN3eGFP-hESCs and their WT counterparts was analyzed by standard cytogenetic procedures. One representative clone of each NGN3eGFP-hESCs line and their WT counterpart were further analyzed by standard cytogenetic procedures having passage numbers of 50 for WT and 60 for transgenic cell lines.

4.8 **Immunocytochemistry of NGN3eGFP$^+$ cells**

Due to the auto-fluorescence and multilayered nature of the day 16 differentiation hESC cultures a thorough optimization of the staining procedure for NGN3 and GFP was needed. The human hepatocarcinoma cell line Huh7.5 (ATCC), transfected with a NGN3-eGFP-Puromycin expression vector, was used to optimize the NGN3 and GFP staining procedure. The NGN3-eGFP-Puromycin cassette, amplified by RT-PCR using day 16 differentiated progeny as template, was cloned into a vector containing the CAGGS constitutive promoter and confirmed by sequencing. Transfection of Huh7.5 cells using lipofectamine 2000 (Life Technologies) yielded a transfection efficiency of around 30%.

Huh7.5 transfected cells and day 16 progeny were fixed with 10% NBF, incubated with 10% donkey serum blocking solution and permeabilized with 0.2% Triton-X100 (Sigma). Optimized staining conditions established by using the NGN3eGFP-Puromycin-expressing Huh7.5 cells as control cells required an amplification step for NGN3 (R&D) which was made possible by using the Tyramide Signal Amplification kit (Perkin Elmer). This was not needed for the staining with the anti-GFP antibody (Abcam).

FACS selected GFP$^+$ cells were stained with an anti-NGN3 antibody (R&D) without need of amplification. DAPI was used as nuclear counterstain in all cases.

4.9 **Fluorescence-activated cell sorting (FACS)**

Cells were detached using accutase (Sigma, A6964) for 5 to 10 minutes at 37°C and were pooled for centrifugation at 500g for 5 minutes. Cells were filtered through a cell strainer of 70µm, again centrifuged and resuspended in PBS (Life Technologies, 20012-019) containing 2% fetal bovine serum (Hyclone, CH30160.03) and 0.1% Penicillin-Streptomycin liquid (Life Technologies, 15140122) for counting using a Neubauer chamber. The sorting experiment was performed on the FacsAriaIII.

For FACS analysis purposes, cells were detached and filtered in the same way. Only prior to fixing with 1% PFA for 10 minutes, cells were washed twice with PBS (Life Technologies, 20012-019) and counted on the Nucleocounter (Chemometec, NC-100). After fixing, cells were washed again twice with PBS (Life Technologies, 20012-019), resuspended in PBS (Life

Technologies, 20012-019) and eventually divided in 500,000 cells per staining condition. After centrifugation, cells were resuspended in 50µl 10% donkey serum (Jackson ImmunoResearch, 017-000-121) with 0.1% Triton-X100 (Sigma, X100) in PBS (Life Technologies, 20012-019). After 15 minutes of blocking, Goat anti-PDX1 (2.5µg/ml, R&D, AF2419) was added for 2 hours at 4°C. Eventually, these cells were washed twice with PBS (Life Technologies, 20012-019) containing 0.1% Triton-X100 (Sigma, X100) and incubated with the secondary antibody Donkey anti-Goat Alexa Fluor 647 (1:500, Life Technologies, A21477) or 488 (1:500, Life Technologies, A31571) for 15 minutes at room temperature and sequentially washed twice with PBS (Life Technologies, 20012-019) containing 0.1% Triton-X100 (Sigma, X100) and resuspended in 200µl of PBS (Life Technologies, 20012-019). Different controls were taken: omit primary and unstained.

4.10 **Immunohistochemistry and FISH**

Kidney grafts were fixed with 4% paraformaldehyde, embedded in paraffin and stained according to standard procedures with 10% of donkey serum blocking solution and 0.2% Triton-X100 (Sigma) permeabilization solution. Staining of the grafts with anti-GFP (Clontech) required amplification of the signal using the Tyramide Signal Amplification kit (Perkin Elmer). This kit was used according to manufacturer's instructions with FITC fluorophores. A list of all antibodies can be found in Supporting Materials Table S3 and S4.

After immunofluorescence staining, grafts were further processed for FISH. The human Cot I DNA probe (Life Technologies) was labeled using the Bioprime Total Genomic labeling system (Life Technologies), diluted in hybridization buffer [6.15g dextrane sulfate, 20ml formamide, 4ml 20xSSC and 18ml water, bring to pH 7 with HCl (all from Sigma)] and denatured at 85°C for 4 min. Hybridization of the slides were performed at 37°C O/N. Thereafter, the slides were washed 2 min. in 0.4x SSC (Life Technologies) with 0.3% Igepal-CA630 (Sigma), 1 min. in 2x SSC with 0.1% Igepal-CA630 and 5 min. in 2x SSC. For the CHGA staining in Figure 18, FISH was performed first, followed by immunostaining. DAPI was used as nuclear counterstain in all the cases.

Imaging of the sorted cells was performed using the Nikon A1R Eclipse Ti, whereas the sections were taken using the AxioImager-Z1.

4.11 *In vitro* c-peptide production

C-peptide release was challenged by incubating the further differentiated eGFP/NGN3-positive clusters in Krebs-Ringer solution with bicarbonate and HEPES (KRBH; 129 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM, KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 10 mM HEPES, 0.1% (wt/vol) BSA, all Sigma). The clusters were washed 3 times using the KRBH. A 30 minutes incubation with KRBH was considered as basal medium; this medium was kept for normalization purposes and was followed by a 1-h incubation with stimulation medium containing either 2.5 mM D-glucose (Sigma, G8270), 20 mM D-glucose, 2.5 mM D-glucose with 1 mM IBMX (Sigma, I5879) and 30 mM KCl (Sigma, P5405) or 20mM D-glucose with 50 μM nifedepine (Sigma, N7634). Samples were analyzed for C-peptide release using the ultra-sensitive C-peptide (Mercodia, 10-1141-01) and C-peptide ELISA kit (Mercodia, 10-1136-01).

4.12 Transplantation under the kidney capsule

Mice (RAG2γc-knock-out mice) were anaesthetized according to the KU Leuven Ethical committee guidelines. The kidney was exposed and a small incision was made in the kidney capsule. An amount of approximately 200.000 cells were pelleted in a poly-ethylene catheter and transferred under the kidney capsule using a Hamilton syringe (Sigma, 20736).

4.13 Statistical analyses

Data values obtained on the differentiation were subject to Student's t-test. Values of $P < 0.05$, $P < 0.01$, and $P < 0.005$ were considered as statistically significant.

4.14 **Tables**

Table 3. Primers used in this study

Primer set	Forward	Reverse
Junction assay (blue)	CCATTCTCTCTTCTTTTCTCCT	GCGGATCTTGAAGTTCACC
Random integration (red)	ATTTAGTGCTTTACGGCACCT	CTCGATGCGATGTTTCGCTT
5’ probe	CAAATAAATTAGCCGGGCACAGTGG	CTAGGGTACAATGCCTGCACGTT
3’ probe	CCTGTCTCGCTGCTTTCG	AATTCCTCACGACACCTGAAATGG
PDX1	TCCACCTTGGGACCTGTTTA	GTGTGTTAGGGAGCCTTCCA
NGN3	TCTCTATTCTTTTGCGCCGG	CTTGGACAGTGGGCGCAC
NEUROD1	CTCGGACTTTTCTGCCTGAG	GTGGAAGACATGGGAGCTGT
OCT4	GATGGCGTACTGTGGGCCC	TGGGACTCCTCCGGGTTTTG
NANOG	CAGCCCCGATTCTTCCACCAGTCCC	CGGAAGATTCCCAGTCGGGTTCACC
MIXL1	GGATCCAGGTATGGTTCCAG	CATGAGTCCAGCTTTGAACC
EOMES	AACAACACCCAGATGATAGTC	TCATAGTTGTCTCTGAAGCCT
CXCR4	GGTGGTCTATGTTGGCGTCT	TGCAATAGCAGGACAGGATG
FOXA2	GGGAGCGGTGAAGATGGA	TCATGTTGCTCACGGAGGAGTA
SOX17	CCTGGGTTTTTGTTGTTGCT	GAGGAAGCTGTTTTGGGACA
INS	ATCAAGCACATCACTGTCCT	TGTAGAAGAAGCCTCGTTCC
GCG	GTTCCCTTCAAGACACAGAG	GGCAATGTTATTCCTGTTCC
SST	GAGGCTTGAGCTGCAGAGAT	TCGCTGAAGACTTGGAGGAT
PAX4	AGCCAAGCAGAGGCACTGG	AACCAGACCCTCACCGTGTCC
ARX	ACAGCGTGTGCCTCTCTGC	TCGGGCCTCGGTCAAGTCC
PAX6	CCCAAGAGCAAATTGAGGCCC	CTCTTCTCCATTTGGCCCTTCGA
NKX2.2	CAACACAAAGACGGGGTTTT	GTTGTCGCTGCTGTCGTAGA
NKX6.1	CCATCTTCTGGCCCGGAGTGA	CTTCCCGTCTTTGTCCAACAA
PTF1A	ACGACTTCTTCACCGACCAG	TGGTGGCTAAGGAACTCCAC

SOX9	ACGCCATCTTCAAGGCGCTG	CCGGCTGCACGTCGGTTTT
HLXB9	ATGATCCTGCCTAAGATGCC	AAATCTTCACCTGGGTCTCG
HNF6	CGCTCCGCTTAGCAGCAT	GTGTTGCCTCTATCCTTCCCAT

Table 4. Primary antibodies used in this study

Antibody	Dilution	Company	Catalog number
Goat anti-OCT4	1:2000	Santa Cruz	SC-8628
Rabbit anti-SOX2	1:500	Milipore	AB5603
Rabbit anti-NANOG	1:150	Abcam	AB80892
Mouse anti-SSEA4	1:500	DSHB	MC-813-70
Mouse anti-TRA-1-60	1:1000	Milipore	MAB4360
Mouse anti-NGN3	1:100	R&D systems	MAB3444
Goat anti-GFP	1:500	Abcam	AB5450
Rabbit anti-GFP	1:200	Abcam	AB290
Rabbit anti-GFP	1:100	Clontech	632376
Goat anti-PDX1	1:250	R&D systems	AF2419
Mouse anti-NKX6.1	1:10	DSHB	F55A10
Guinea pig anti-INS	1:200	Dako	A0564
RabbitMab antiGCG	1:200	Abcam	2752
Goat anti-SST	1:500	Santa Cruz	SC-7819
Mouse C-PEPTIDE	1:250	Abcam	AB93903
Mouse anti-CHGA	1:1000	Biogenex	LK2H10
Mouse anti-CD142	1:10	BD Biosciences	561713
Mouse anti-CK19	1:200	Dako	M0888
Rabbit anti-AMYLASE	1:200	Thermo Scientific	PA5-22245
Mouse anti-GFAP	1:200	Sigma	G3893
Mouse anti-CDX2	1:500	Biogenex	CDX2-88
Rabbit anti-MUCIN-2	1:1000	Santa Cruz	SC-15334
Rabbit anti-LYSOZYME	1:1500	Dako	A0099
Mouse anti-ALB	1:50	R&D systems	MAB1455

Mouse anti-TUJI1	1:1000	Eurogentec	PRB-453P
Mouse anti-smooth muscle actin	1:200	Sigma	A2547

Table 5: Secondary antibodies used in this study

Antibody	Dilution	Company	Catalog number
Donkey anti-RabbitCy5	1:500	Jackson ImmunoResearch	711-175-152
Donkey anti-MouseCy5	1:500	Jackson ImmunoResearch	715-175-150
Donkey anti-GoatAlexaFluor®555	1:500	Life Technologies	A21432
Donkey anti-MouseAlexaFluor®555	1:500	Life Technologies	A31570
Goat anti-Guinea pig AlexaFluor®488	1:500	Life Technologies	A11073

Results described in this chapter have been taken and partially adapted from:

Cai, Q., Bonfanti, P., Sambathkumar, R.*, Vanuytsel, K., Vanhove, J., Gysemans, C., Debiec-Rychter, M., Raitano, S., Heimberg, H., Ordovas L.** and Verfaillie C.** (accepted Stem Cells Translational Medicine)* *, **These authors contributed equally.* ***Prospectively isolated human NGN3-expressing progenitors give rise to endocrine hormone-expressing cells***

5 RESULTS

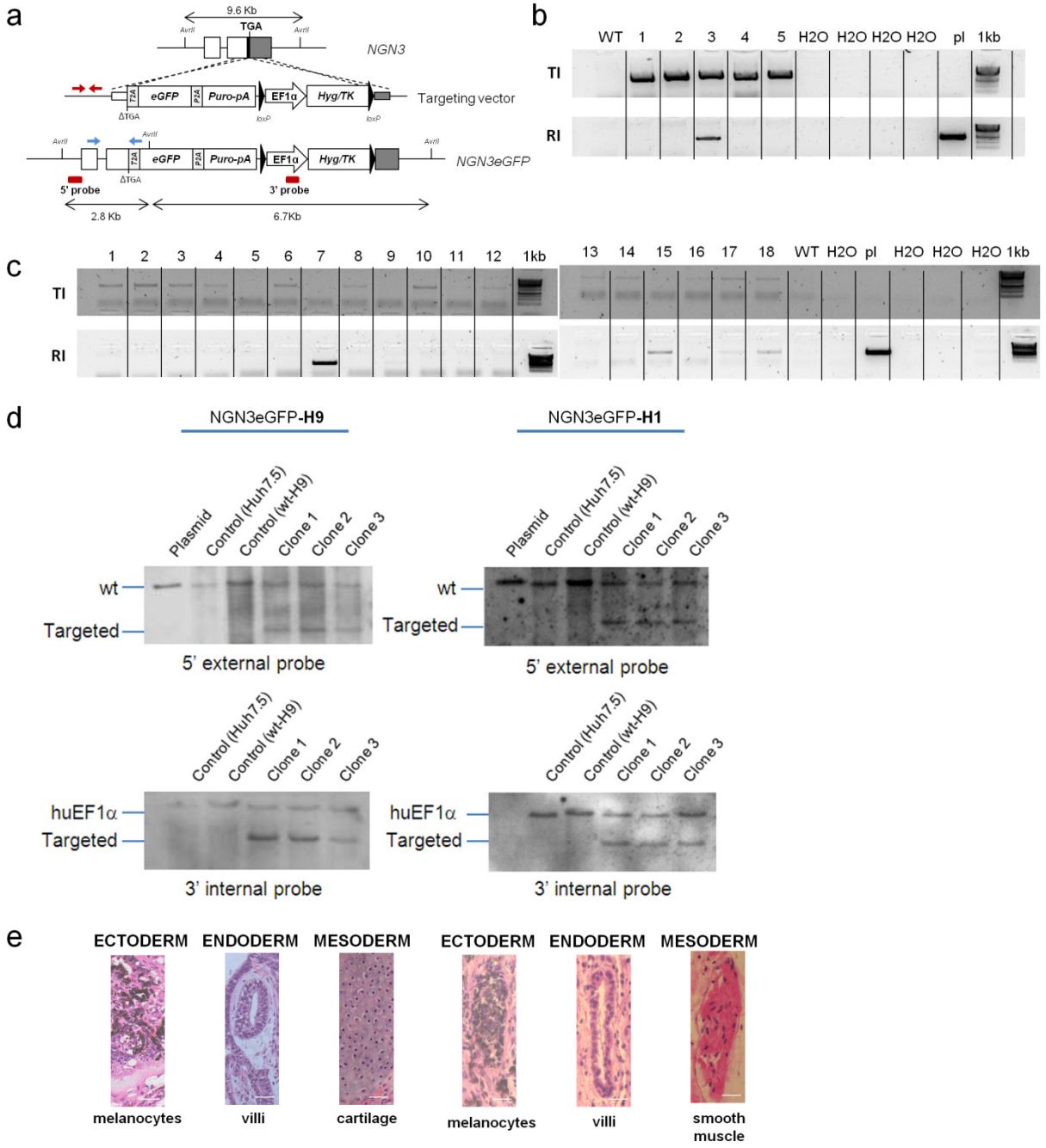
5.1 **Generation of NGN3eGFP-hESC lines**

ZFNs were generated to recognize unique sites near the stop codon of the *NGN3* gene. A gene targeting vector containing isogenic homology regions of up to 800bp was created. Upon correct targeting, the stop codon becomes deleted and *NGN3* becomes tagged with a polycistronic cassette constituted of an enhanced green fluorescent protein (eGFP) and a puromycin resistance antibiotic ($Puro^R$) linked by T2A and P2A (Figure 7a, see Appendix for nucleotide sequence). The self-cleaving peptides (T2A and P2A) lead to the production of three individual proteins once NGN3 is expressed. An EF1α (Elongation Factor 1 alpha) -$hygromycin^R$/thymidine kinase (Hyg/TK) fusion cassette allows the selection of targeted clones.

After nucleofection of the ZFNs and the donor plasmid in H9- and H1-hESCs, antibiotic selection was performed and respectively 18 and 5 hygromycin-resistant colonies were manually picked. Genotyping polymerase chain reaction (PCR) was performed to identify the correctly targeted clones: 12/18 H9 and 4/5 H1 were targeted without random integrations (Figure 7b, c). For the amplification of the targeted allele we used the primer set depicted as blue arrows in Figure 7a, whereas for the detection of random integrants we used the primer set depicted as red arrows in Figure 7a. Of these clones, we choose three of each hESC line for further characterization. Southern blot analysis confirmed the targeted integration of our reporter construct without random integrants (Figure 7d).

Genome editing based on HR with ZFNs is known to cause off-target mutations, which may affect ESC characteristics. Therefore the genetically modified cell lines were assessed for

maintenance of pluripotency and genome integrity. The targeted lines formed teratomas wherein cells of the three germ layers, endoderm, mesoderm and ectoderm, could be detected (Figure 7e) and expressed the pluripotency markers OCT4, NANOG, SOX2, TRA-1-60 and SSEA4 (Figure 7f).



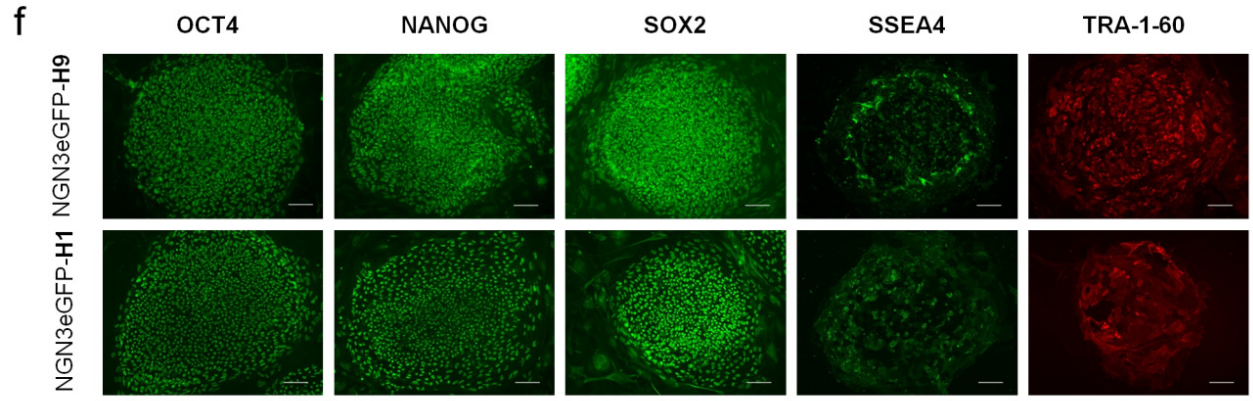
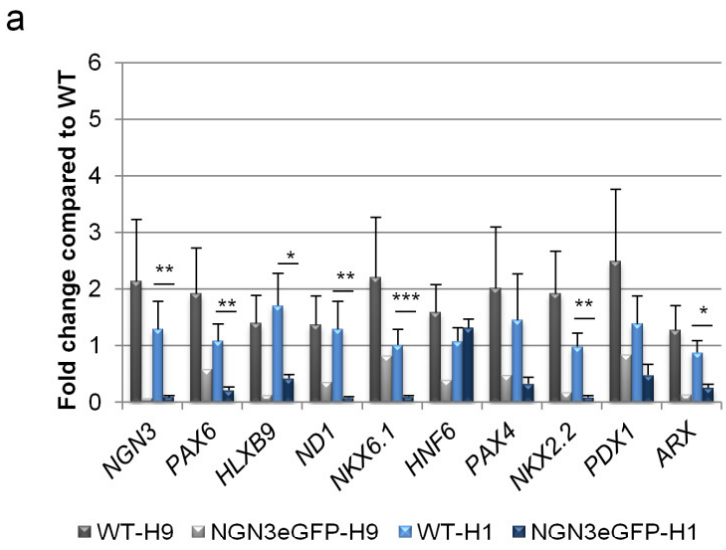
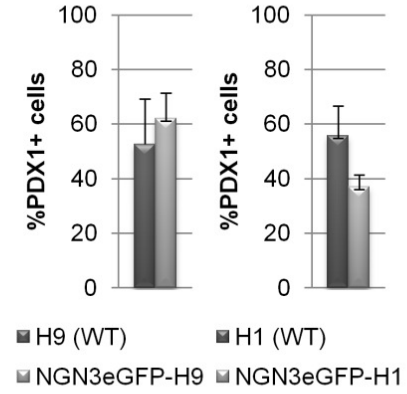
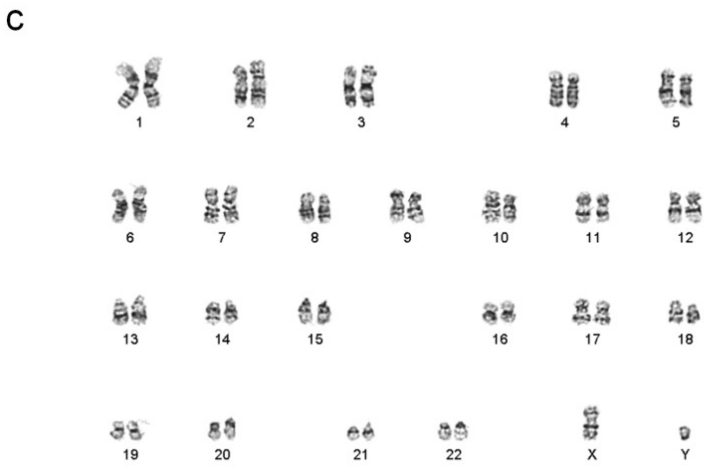


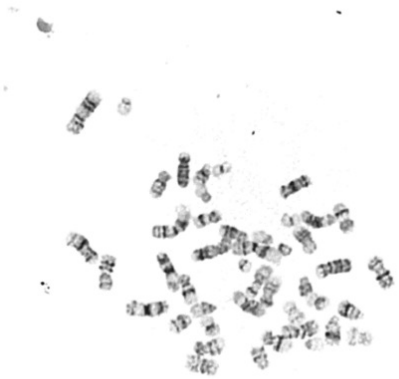
Figure 7 Characterization of the NGN3eGFP-hESC lines (a) Schematic overview of WT allele, targeting vector and targeted NGN3 alleles. Red lines, probes used in southern blot; white boxes, coding sequences; black line, ZNFs genomic recognition site; dark grey, 3'UTR; light grey boxes, T2A and P2A sequences; eGFP and PURO-pA (PuromycinR gene and polyadenylation signal); white arrow, EF1αpromoter (human elongation factor 1 alpha promoter); Hyg/TK, hygromycinR-thymidin kinase fusion gene. PCR genotyping primers are indicated in blue (amplification of the integration) and red (amplification of random integrants). (b) PCR-genotyping of five H1 hygromycin-resistant clones. First panel shows PCR for targeted integration (TI) using the primers indicated by the blue arrows. Second panel shows PCR for randon integration (RI) using the primers indicated by the red arrows (c) PCR-genotyping of eighteen H9 hygromycin-resistant clones using the same primers as for the H9 hygromycin-resistant clones. (d) Southern blot of NGN3eGFP-hESC lines. Genomic DNA was digested with AvrII and hybridized with external (5') and internal (3') probes. (e) Hematoxylin and eosin staining of teratoma sections generated from the NGN3eGFP-hESC lines representing all three lineages. (f) Immunostaining of the NGN3eGFP-hESC lines for pluripotency markers OCT4, NANOG, SOX2, SSEA4 and TRA-1-60. Scale bars 100μm.

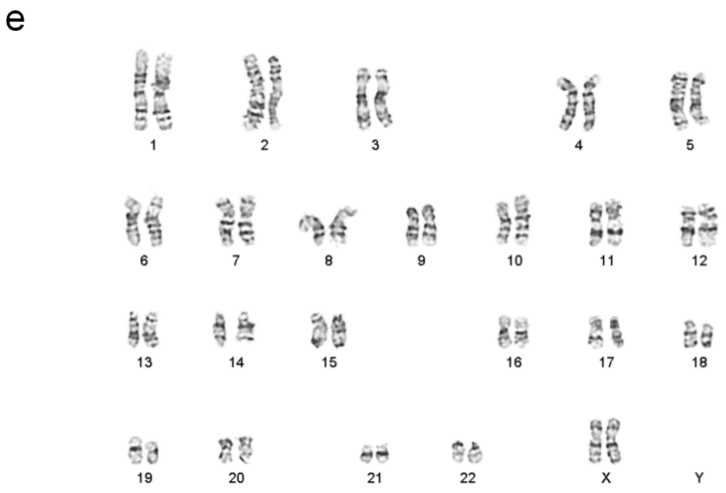
The transgenic cell lines were also assessed for their *in vitro* differentiation potential by directing the cells towards pancreatic endoderm as described in the next paragraph. No significant differences in the transcript levels of *HLBX9*, *HNF6*, *PDX1*, *NGN3*, *NEUROD1*, *PAX4*, *ARX*, *PAX6*, *NKX6.1* and *NKX2.2* were observed between wild type (WT) and NGN3eGFP-H9 day 16 progeny (Figure 8a). We also compared the number of PDX 1^+ cells generated in both cell lines using FACS (Figure 8b). This confirmed that the targeted cell lines retained full differentiation potential after HR. Western blot experiments were performed using protein of differentiated day 16 hESC (WT and transgenic) progeny, unfortunately no positive signal could be detected for NGN3, which could be due to the low amount of $NGN3^+$ cells (0.2-2%) in the cultures. Genome integrity was assessed by both aCGH, due to the higher sensitivity to detect copy number variations than traditional cytogenetic techniques, and conventional karyotyping. aCGH performed on three different clones demonstrated that no genetic abnormalities were present after ZFN treatment. The karyotype and ploidy of both NGN3eGFP-hESC lines were normal (Figure 8c-f).


a
Fold change compared to WT
0
1
2
3
4
5
6
**
**
*
**
***
**
*
NGN3
PAX6
HLXB9
ND1
NKX6.1
HNF6
PAX4
NKX2.2
PDX1
ARX
WT-H9
NGN3eGFP-H9
WT-H1
NGN3eGFP-H1


b
%PDX1+ cells
0
20
40
60
80
100
%PDX1+ cells
0
20
40
60
80
100
H9 (WT)
H1 (WT)
NGN3eGFP-H9
NGN3eGFP-H1


c
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y


d


e
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y


f

Figure 8 Assesment of differentiation potential, karyotype and ploidy of NGN3eGFP-hESCs (a) Transcript levels of pancreatic endocrine genes of NGN3eGFP-hESC lines compared to WT lines. (b) Percentage of $PDX1^+$ cells on day 16 of the differentiation also compared to WT lines. The NGN3eGFP-hESCs were analyzed using Giemsa banding (c) shows the karyotype and (d) the ploidy of NGN3eGFP-H1 (e) shows the karyotype and (f) the ploidy of NGN3eGFP-H9. All results shown are from three independent experiments. Data are represented as mean ± SEM.

5.2 **Differentiation of hESCs towards endocrine pancreas**

We generated a protocol to differentiate hESCs towards endocrine pancreas (see Figure 9a). Activin A and Wnt3a were used for induction of definitive endoderm (DE)[92, 113]. We next used a mix of anti-SHH antibody, to inhibit Sonic Hedgehog Signaling (SHH)-signaling[93, 122, 123]; retinoic acid (RA) to direct ESCs towards endocrine pancreas by inducing *PDX1* expression[92, 124]; NOGGIN to inhibit BMP signaling[113, 124] and nicotinamide (NA) which is known to induce differentiation and maturation of undifferentiated fetal pancreatic cells[125, 126].

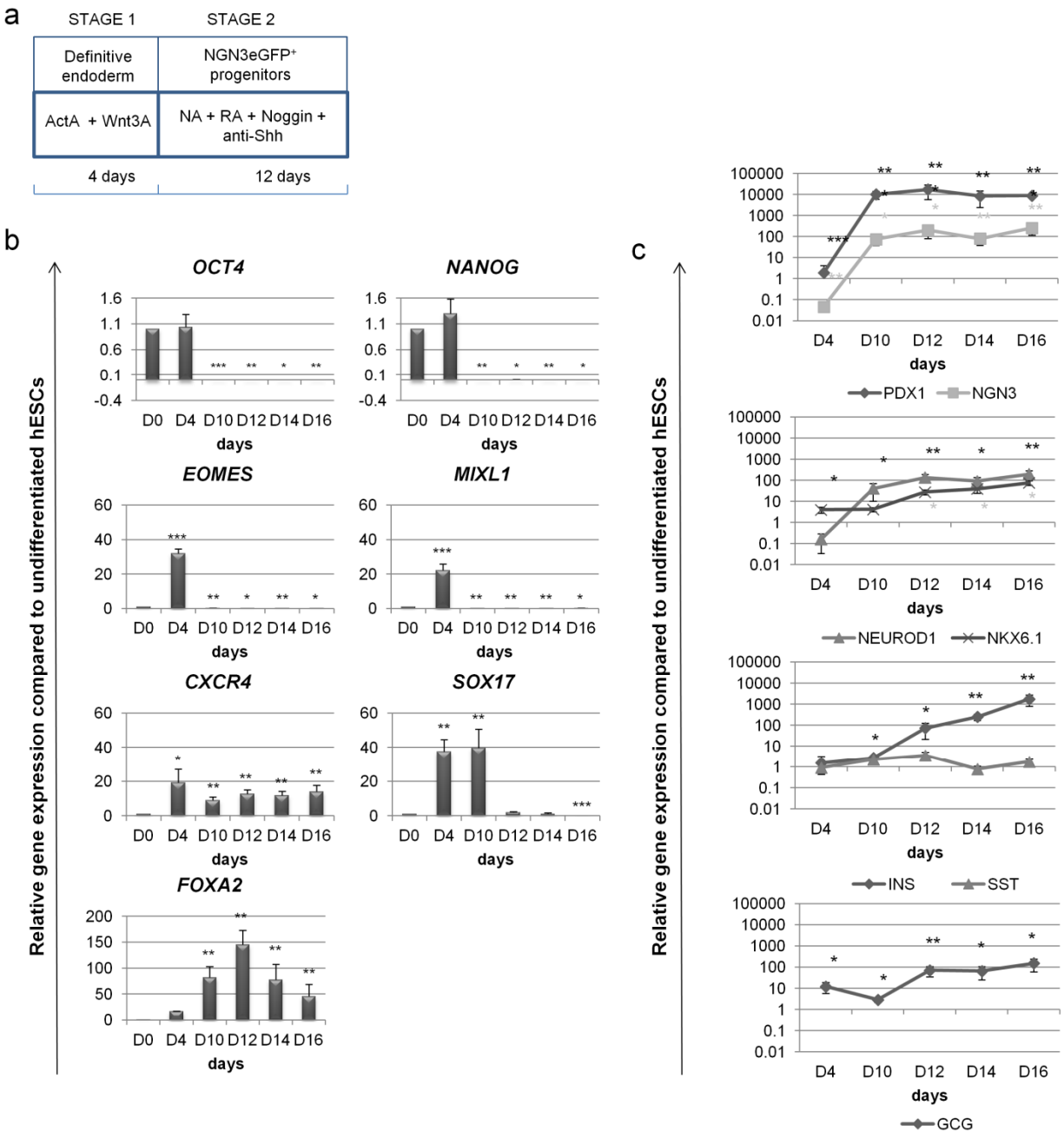


Figure 9 Generation of NGN3eGFP$^+$ cells from the NGN3eGFP-hESC lines (a) The differentiation scheme depicts the mix of factors used to commit hESCs towards endocrine pancreas.(b) Gene expression of several transcription factors during the differentiation shown in bars. (c) Transcript levels for pancreas specific genes *PDX1*, *NGN3*, *NEUROD1*, *NKX6.1*, *INS*, *SST* and *GCG* also during endocrine pancreas differentiation shown in line graphs. All results shown are from three or more independent experiments.Data are represented as mean ± SEM.

We evaluated the differentiation process by RT-qPCR on day 0, 4, 10, 12, 14 and 16. Transcript levels for the pluripotency genes *OCT4* and *NANOG* decreased from d4 onwards and were, as

expected, no longer expressed following day 10. Transcripts of the primitive streak (PS) marker genes (*EOMES* and *MIXL1*) were maximally expressed on day 4 and transcripts for the DE genes (*CXCR4*, *FOXA2* and *SOX17*) increased from day 4 to day 10, but decreased afterwards (Figure 9b) consistent with a transient commitment of endoderm-specific cells in our cultures. From day 4 onwards, we could detect transcripts for the pancreatic endocrine gene *PDX1*, *NGN3* and *NEUROD1* (Figure 9c). *INS* and *GCG* transcript levels increased moderately and *SST* was not expressed as compared to undifferentiated cells, which did not express any of these pancreactic genes (Figure 9c). These expression levels remained respectively ~9000, ~2000 and ~80000 fold lower than in human pancreas (data not shown). When this protocol was used to differentiate the H1-hESC line, gene expression of pancreatic endocrine genes was different on day 16 for the same genes (Figure 10), reflecting the common observation that the differentiation ability of different human ESC lines towards a specific lineage differs [92, 113, 124, 127].

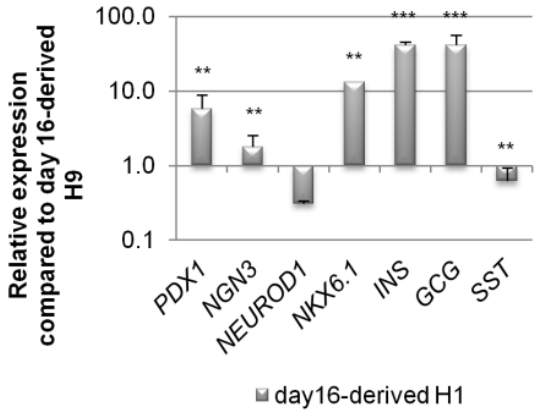
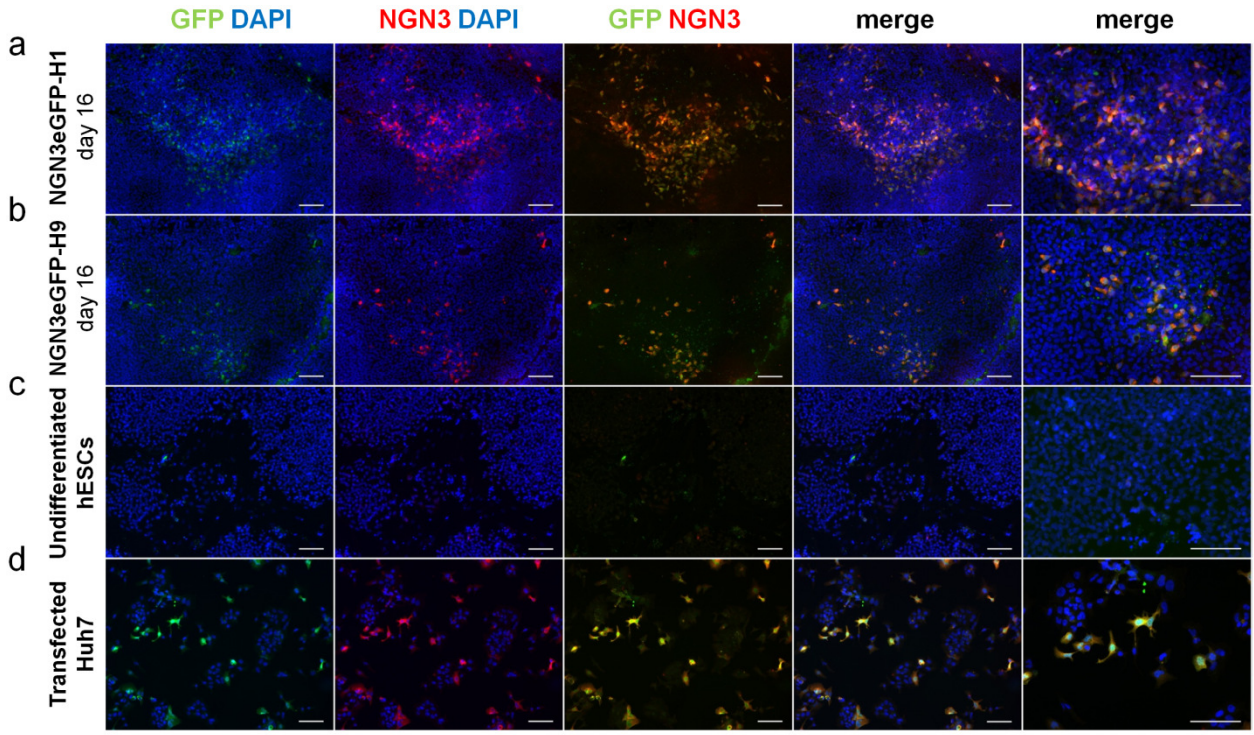


Figure 10 Comparison of wt-H9 with wt-H1 cells on day 16 of the pancreatic endocrine differentiation Relative expression values for *PDX1*, *NGN3*, *NEUROD1*, *NKX6.1*, *INS*, *GCG* and *SST* for day 16 H1-progeny compared to day 16 H9-progeny. All results shown are from three or more independent experiments.Data are represented as mean ± SEM.

As robust *NGN3* expression could be detected between day 12 and day 16, we choose day 12 for the initiation of the puromycin selection and day 16 to isolate committed $NGN3^+$ cells from the targeted lines. In order to determine the faithfulness of the knock-in add-on, we analyzed GFP and NGN3 expression on non-selected day 16 progeny of NGN3eGFP-H9 and -H1 expressing cells. The specificity of both antibodies was first tested on positive control Huh7.5 cells (Figure 11d and g) to eventually demonstrate that the GFP signal co-localizes consistently with the

NGN3 expression in the transgenic lines (Figure 11a, b, e and f). Some studies have suggested that retention of the cassette used for the selection of recombinant clones may interfere with the faithfulness of the lineage specific reporter cassette[100, 128]. However, in day 16 progeny of NGN3eGFP-H1 and -H9 cell lines, all GFP^+ cells were $NGN3^+$ and all $NGN3^+$ cells were GFP^+, demonstrating that the reporter lines faithfully express GFP from the endogenous *NGN3* locus and ruling out the need to remove the selectable cassette.



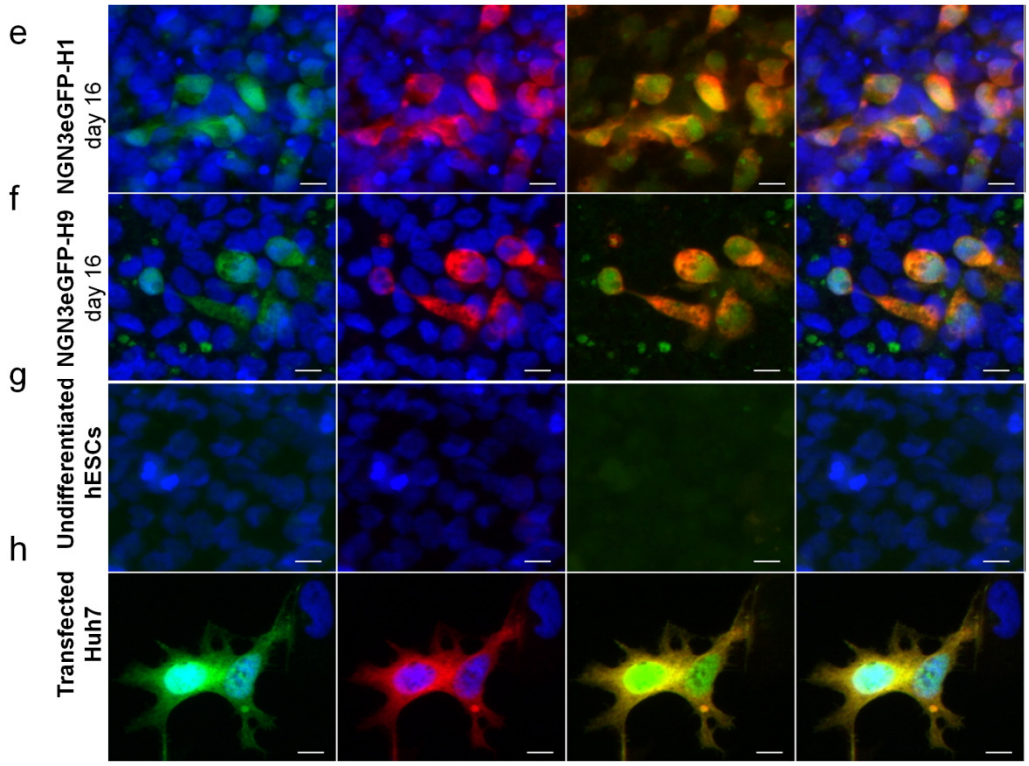
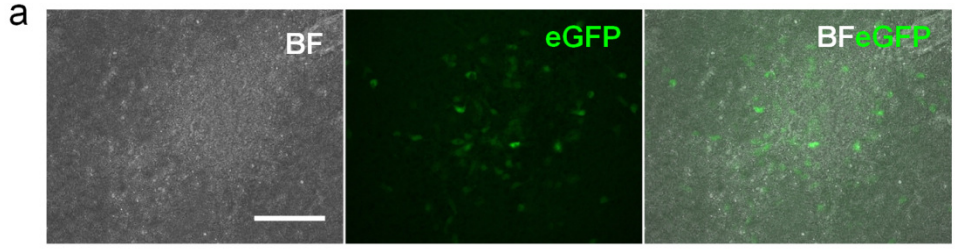


Figure 11 NGN3 and GFP immunocytochemistry of NGN3eGFP-hESCs-derived day 16 progeny. (a, b) Day 16 NGN3eGFP-hESCs-derived cells (unsorted and non-selected), (c) undifferentiated hESC and (d) NGN3-GFP transfected Huh7.5 cells were stained with anti-NGN3 to confirm the co-localization of NGN3 and GFP. Scale bars 100µm. (e, f) Day 16 NGN3eGFP-hESCs-derived cells (unsorted and non-selected), (g) undifferentiated hESC and (h) NGN3-GFP transfected Huh7.5 cells were stained with anti-NGN3 to confirm the co-localization of NGN3 and GFP. Scale bars 10µm.

To isolate committed NGN3eGFP$^+$ cells (Figure 12a), targeted lines were used to select NGN3-expressing cells on day 16 as *NGN3* mRNA expression is detectable from day 10 onwards (Figure 9Figure 13c). The differentiating hESCs were treated with 600ng/ml of puromycin between d12 and d16 to pre-enrich the NGN3eGFP$^+$ fraction (ranging between 0.3 to 2%) before FACS. To ensure the selection of highly purified cells and due the low amount of NGN3eGFP$^+$ cells in our differentiations, two sequential sorts were performed (Figure 12b).



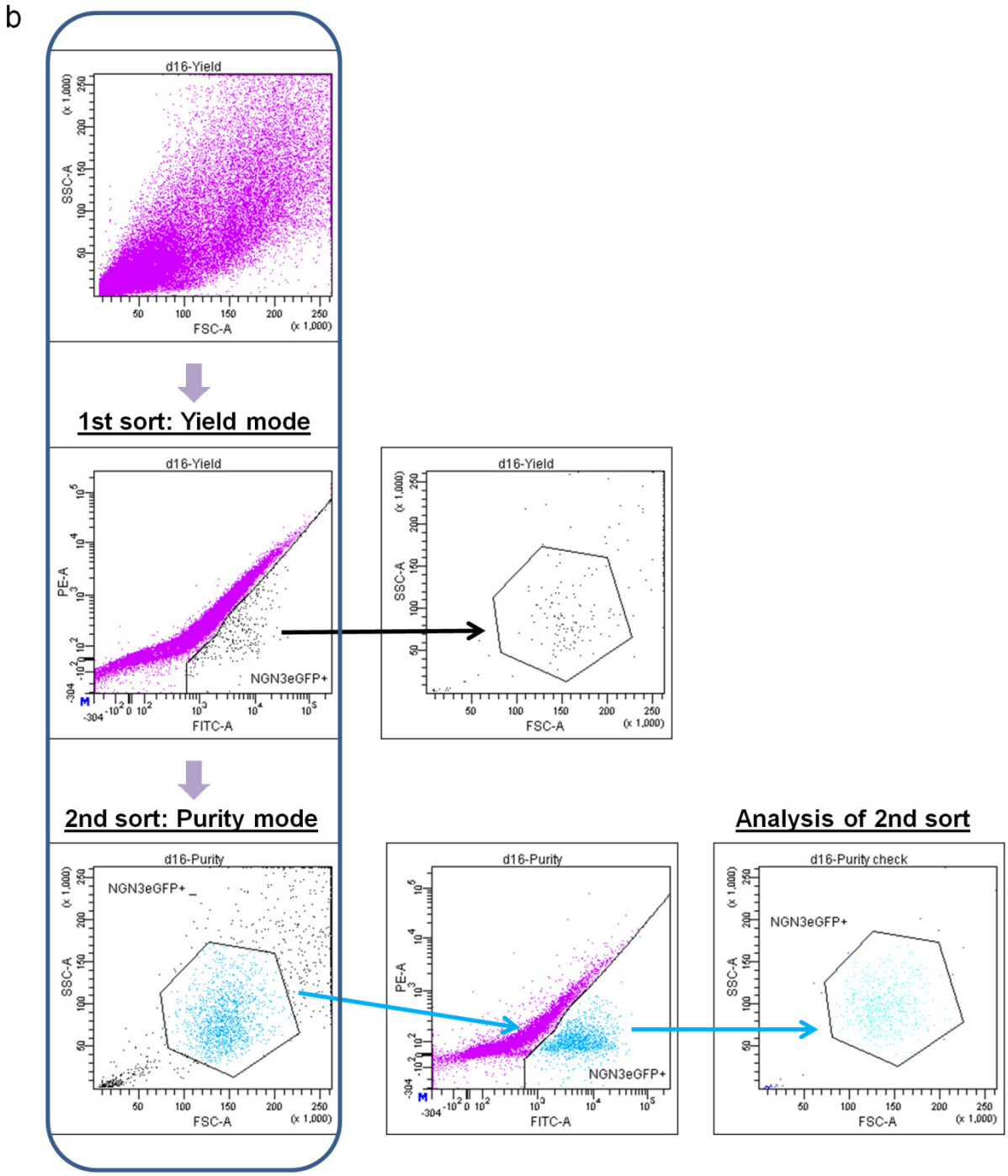


Figure 12 (a) Bright field (BF), eGFP and overlay image (BFeGFP) of puromycin-selected differentiation cultures (live image). Scale bar 100µm. (b) FACS plots showing the sorting strategy to enrich for NGN3eGFP^{+} cells. The NGN3eGFP^{+} cells were first sorted in yield mode, followed by purity sort according to their forward and side-scatter profile.

The first sort was performed in yield modus which allows a fast and efficient though less specific selection, whereas the second sort was performed in purity modus which ensured a highly purified end-population. The final purity of the FACS-selected NGN3eGFP$^+$ cells was 90.9% ±2.5 (n = 3), the remaining ~10% can be mostly accounted as cell debris as determined by their low forward and side scatter (Figure 12b last panel with analysis of the second sort).

NGN3 mRNA was 63-fold more abundant in this cell population relative to unsorted cells (Figure 13). The small number of NGN3$^+$ cells that could be selected by FACS is consistent with the difficulties we had concerning the detection of NGN3 protein by western blot within the unsorted cell population. Obviously, this suggests there is still room for improvement of the differentiation protocol to generate larger numbers of NGN3$^+$ cells. The availability of the NGN3 lineage tracing line, described here should now allow larger throughput screens to identify factors that enhance differentiation towards NGN3$^+$ endocrine pancreas cells, as has for instance been described by Melton's group (2012)[99].

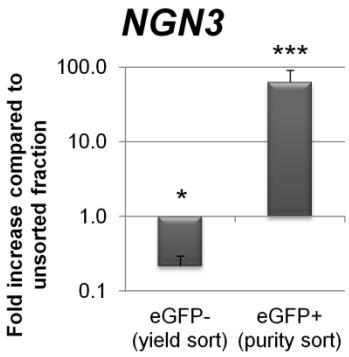


Figure 13 NGN3 expression profile of the NGN3eGFP$^-$ and the NGN3eGFP$^+$ fraction after sorting on d16 of the differentiation NGN3 mRNA level was 63-fold more abundant in the NGN3eGFP$^+$ population compared to the NGN3eGFP$^-$ population, both relative to unsorted cells. All results shown are from three or more independent experiments. Data are represented as mean ± SEM.

5.3 **Characterization of the NGN3eGFP$^+$ cells from differentiating hESCs**

NGN3eGFP$^+$ cells were evaluated by RT-qPCR and immunostaining. In addition to the significantly increased transcript levels for *NGN3,* other pancreatic marker genes (*PDX1*, *NEUROD1*, *PAX4*, *ARX*, *PAX6*, *NKX2.2*, *NKX6.1*, *INS*, *SST* and *GCG*) were significantly enriched compared with the unsorted fraction (Figure 14a and b).

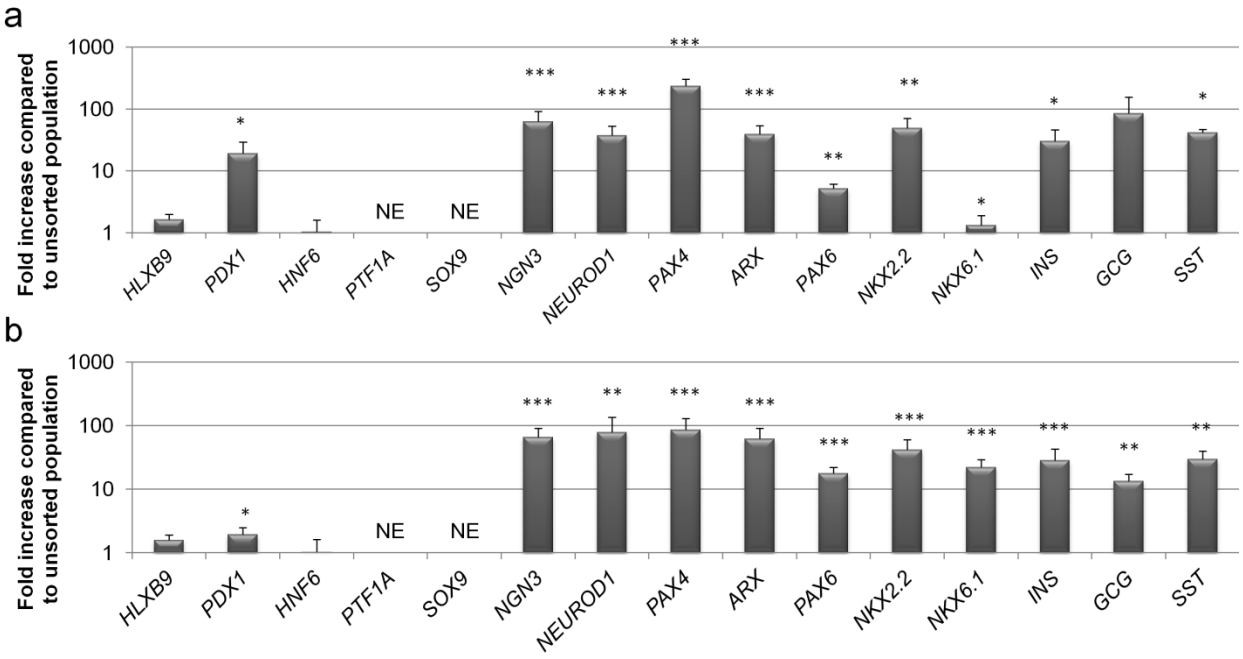
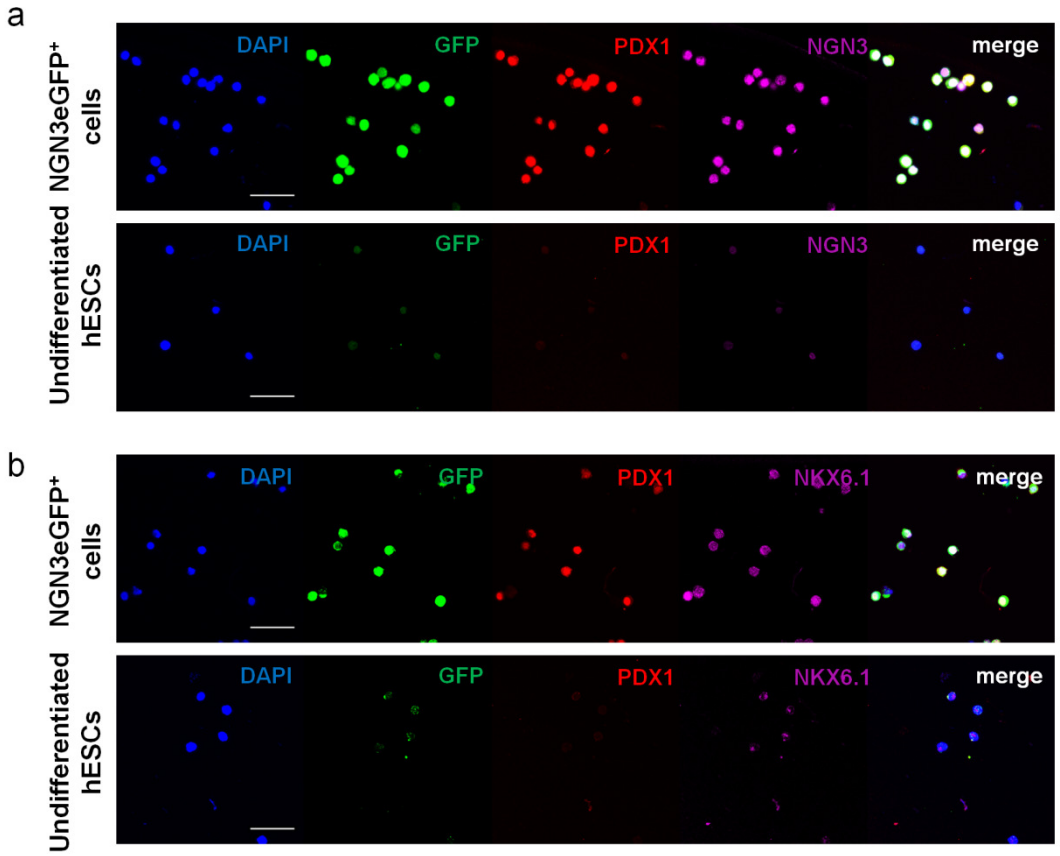


Figure 14 Gene expression profile of the sorted NGN3eGFP$^+$ cells from H9- and H1-progeny Several important pancreatic transcription factors are more abundantly expressed in the NGN3eGFP$^+$ fraction of differentiating (a) H9 and (b) H1 cells compared to the unsorted fraction. All results shown are from three or more independent experiments. Data are represented as mean ± SEM.

Presence of *INS, SST* and *GCG* transcripts in NGN3eGFP$^+$ progenitors may not be readily explained. However, immunostaining demonstrated that NGN3eGFP$^+$ cells stained only very faintly with antibodies against INS, GCG and SST (Figure 15d). A similar mRNA expression profile has been described by Xu *et al.* (2008) for the most committed subpopulation of Ngn3-expressing cells (eGFP$^+$-high side scatter, eGFP-HSSC) isolated from adult mouse pancreas after partial duct ligation[54]. Like mouse eGFP-HSSCs, human NGN3eGFP$^+$ cells did not express *SOX9* or *PTF1a* transcripts. *PTF1a* is not required for *NGN3* specification[129], while *SOX9* is expressed in early multi-potential progenitors of the pancreas during development[34], indicating that the NGN3eGFP$^+$ cells may already have passed through that stage. *HLXB9* and *HNF6*

transcripts were present in NGN3eGFP$^+$ cells but at levels similar to those in the unsorted population (Figure 14a and b), likely because of the upstream and more widespread expression of these genes[130]. All eGFP$^+$ cells stained positive for NGN3, PDX1 and NKX6.1 (Figure 15a and b), further confirming their progenitor state. In addition NGN3eGFP$^+$ cells stain positive for chromogranin-A (CHGA) (Figure 15c), but were negative for CD142 (data not shown). CHGA was recently shown to be absent in pancreatic endodermal progenitors, but present on endocrine cells derived from hESCs[101]. The presence of CHGA could point out towards a novel characteristic of NGN3$^+$ cells in humans as it is usually linked to mature endocrine cells, although more investigation will be needed to confirm this observation. CD142 was used by Kelly *et al.* (2011) as a surface marker for pancreatic endoderm, these cells have the potential to generate all three pancreatic lineages (endocrine, exocrine and ductal cells)[101].



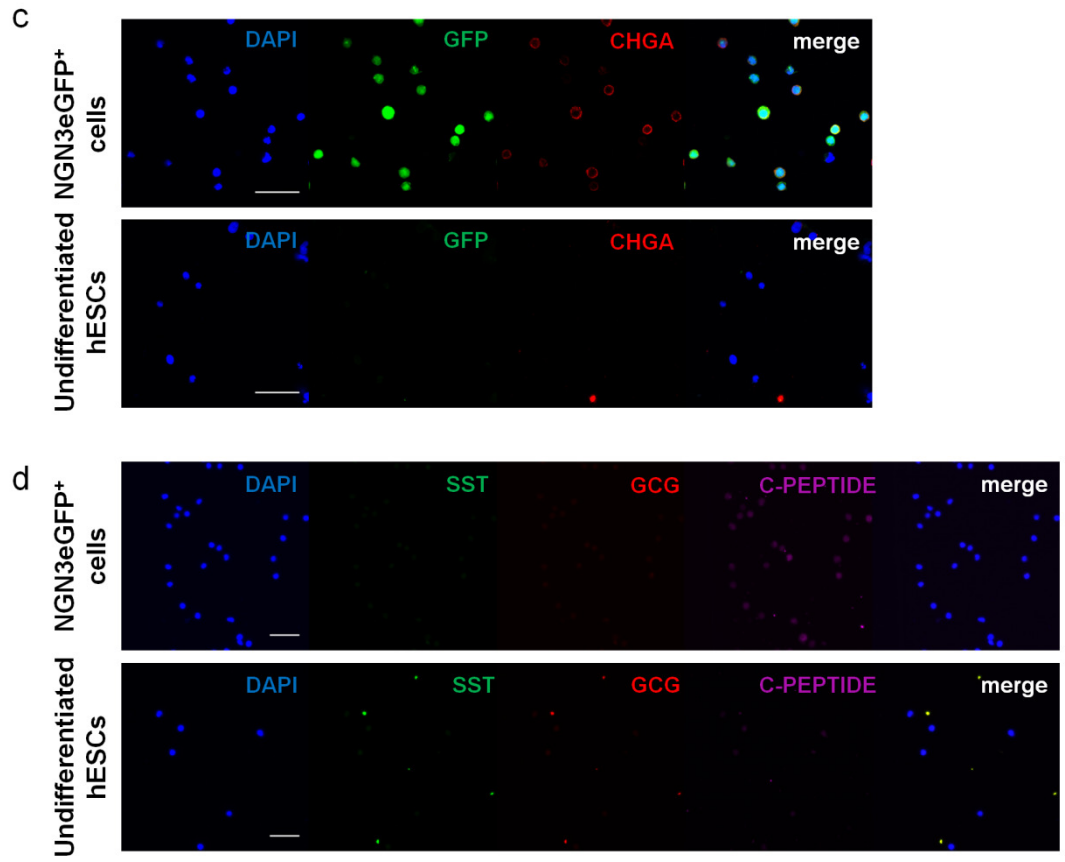


Figure 15 Immunocytochemistry of the sorted NGN3eGFP$^+$ cells (a-c) NGN3eGFP$^+$ cells express GFP, PDX1, NGN3, NKX6.1 and CHGA. (d) A very faint positive signal could be seen for C-peptide, but no positive staining could be detected for SST or GCG and. Scale bars (a-c) 25µm and (d) 50µm.

To induce further differentiation of the NGN3eGFP$^+$ cells to pancreatic endocrine hormone-producing cells, we plated NGN3eGFP$^+$ cells in low attachment plates to allow cell cluster formation. The clusters were maintained for an additional 24 days with insulin-like growth factor-II (IGF-II) and NA, a combination of factors previously shown to support *in vitro* maturation of progenitors to INS$^+$ cells[126]. Transcript levels for *NGN3* were, as expected with maturation, significantly down regulated while expression levels of *NKX6.1* and the endocrine hormones, *INS*, *GCG* and *SST* were significantly increased (Figure 16a).

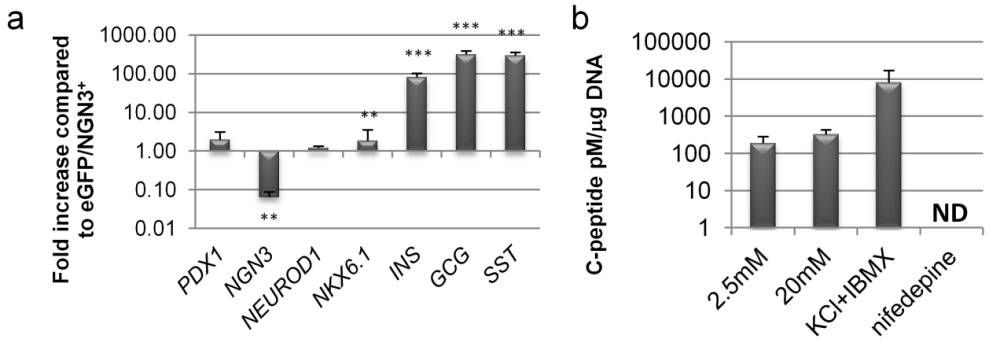


Figure 16 NGN3eGFP$^+$-derived clusters cultured for 24 days with IGFII and NA (a) mRNA expression levels of pancreatic markers and (b) These clusters were challenged with 2.5mM, 20mM glucose, 2.5mM D-glucose with 1mM IBMX and 30mM KCl or 20mM D-glucose with 50µm nifedepine to allow C-peptide release. ND: not detected. All results shown are from three or more independent experiments. Data are represented as mean ± SEM.

We assessed the glucose responsiveness of the NGN3eGFP$^+$ derived clusters. Exposure to 20mM D-glucose did not induce secretion of C-peptide. However, stimulation with KCl and the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX) induced C-peptide release. On the other hand nifedipine, a calcium antagonist, blocked the basal C-peptide release seen with 20mM glucose-stimulation (Figure 16b). These findings are in accordance with other published reports [92, 120, 126] and may reflect the significantly lower levels of *INS* expression in the NGN3eGFP$^+$ clusters compared with islets of Langerhans. As mentioned in the introduction, little is known about *in vitro* maturation of pancreatic endocrine cells. According to a recent publication from the Sander's group (2013), this could be due to the lack of correct histone modification marks during endocrine differentiation *in vitro*, which is not the case when the cells are allowed to mature *in vivo*. Endocrine cells generated *in vivo* more closely resemble human islets with regards to their transcriptome and chromatin structure[115].

5.4 NGN3eGFP$^+$ cells have the capacity to differentiate towards endocrine hormone-expressing cells *in vivo*

Approximately 200,000 NGN3eGFP$^+$ cells were transplanted immediately after sorting under the kidney capsule of normoglycemic immunodeficient mice to assess if further maturation occurs *in vivo*. Mice were sacrificed after 8-9 weeks and kidneys harvested. Grafts were present in all transplanted mice and human cells could be identified by fluorescence *in situ* hybridization (FISH) using a specific human DNA CotI probe (Figure 17).

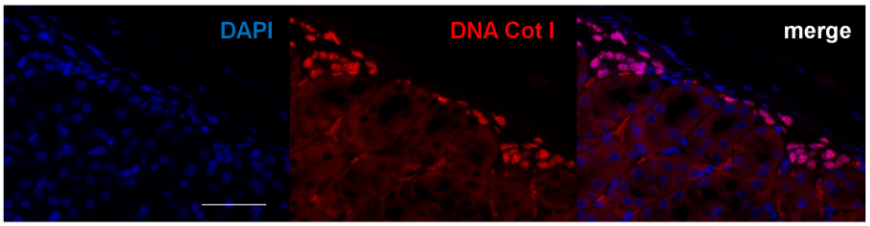


Figure 17 FISH of the engrafted NGN3eGFP+ cells after 8-9 weeks of in vivo maturation Human cells could be visualized using a fluorescently labeled DNA cotI probe. Scale bar 100µm.

Human cells positive for CHGA were detected (Figure 18a). Some of the CHGA$^+$ cells stained positive for INS, GCG or SST (Figure 18b-d, f, g). Even though some authors have noted INS$^+$/GCG$^+$ and INS$^+$/SST$^+$ cells in hESC-derived grafts[92, 119], no double hormone positive cells were found in the grafts. Neither NGN3eGFP$^+$ could be detected (Figure 17e). The exocrine marker Amylase or the ductal marker CK19 was not detected. As NGN3-expressing cells can also give rise to glial precursor cells[131], we tested if the NGN3eGFP$^+$-derived cells stained positive for GFAP, but did not find positive cells. Other studies demonstrated that NGN3$^+$ cells can differentiate into intestine and gut[132, 133]. However, NGN3eGFP$^+$ progeny in this study did not stain positive for the intestinal markers CDX2, MUCIN-2 and LYSOZYME. The grafted cells were also negative for the liver marker albumin, the neuronal marker β-tubulin-III and the mesodermal marker alpha-smooth muscle actin (data not shown). Teratomas were not detected. Many cells in the graft remained unidentified, as they stained positive for the DNA cotI probe, but not for any of the above mentioned proteins. Most transplantation studies performed with pancreatic progenitor populations make use of enriched cell cultures[95, 101, 119]. To our knowledge, this is the first report using highly purified progenitor cells without prior expansion. As the cells

were grafted under the kidney capsule the possibility remains that if the cells were grafted in a more ideal environment, more robust differentiation towards endocrine cells might be possible. For example the aggregation of our NGN3eGFP^{+} cells with fetal pancreata of unborn mice transplanted under the kidney capsule, could aid in a more efficient differentiation of human NGN3eGFP^{+} cells towards islet cells. We attempted this approach, using 30,000 NGN3eGFP^{+} cells. Unfortunately this assay needs larger number of cells (>500,000 cells) to ensure that enough cells aggregate with the mouse pancreas. Alternatively, supporting cells could be needed to direct all cells in an efficient way towards pancreatic endocrine cells as was done before for SOX17^{+} cells[100]; or certain growth factors may need to be added as suggested by Yechoor *et al.* 2009 for liver cell reprogramming[60].

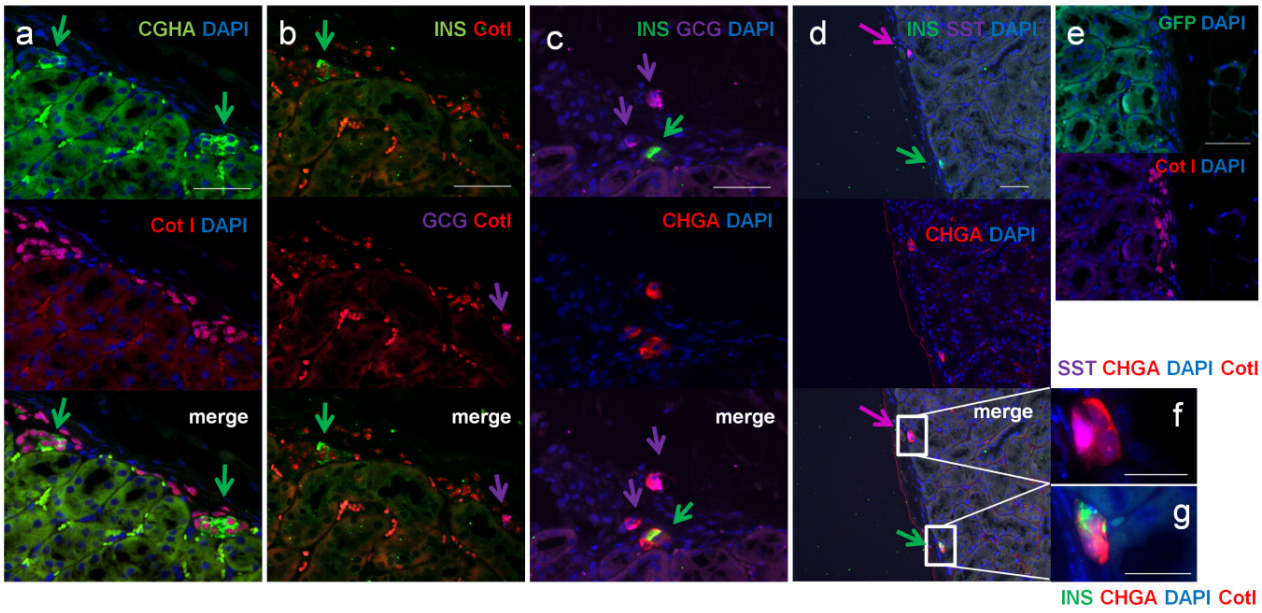


Figure 18 NGN3eGFP^{+}cells transplanted under the kidney capsule give rise to endocrine-positive progeny (a-e) FISH with labeled CotI DNA and/or immunostaining for CHGA, INS, GCG, SST and GFP. Human CHGA+ cells are indicated in (a) green arrows. Human INS+ and GCG+ cells are shown in (b), respectively green and purple arrows. Single-positive INS (green arrows), GCG (purple arrows) and SST (magenta arrows) cells co-express CHGA (c, d). NGN3eGFP+ cells were not detected in the graft (e). (f, g) Magnification of CotI, CHGA and SST (e) and CotI, CHGA and INS (f). Scale bars 50µm (a-c, e), 100µm (d), 20µm (f, g).

6 DISCUSSION

Many attempts have been made to generate pancreatic β-cells from pluripotent stem cells. An important hurdle that investigators generally face is the fact that most of these directed differentiations generate mixed progeny[92, 95, 119]. Here we used ZFN-mediated HR to knock-in an eGFP-Puro^R cassette in the *NGN3* gene in hESCs. After further analyses of the targeted hESCs, we can conclude that the targeting experiment did not affect pluripotency. Most gene targeting studies in hESCs have disrupted one copy of the gene of interest[100, 112] by introducing a selection cassette in the initial coding exons. Abrogation of the expression of one allele may cause haploinsufficiency of the gene of interest, which could be important for key transcription factors as it has been shown for mouse Oct4 [134]. To retain both *NGN3* alleles intact, we choose to insert a polycistronic 2A-linked reporter-selectable cassette at the C terminus of *NGN3,* deleting the stop codon. This allowed the selection of pure NGN3$^+$ cells from mixed hESC progeny. To our knowledge, this is the first detailed study reporting a knock-in add-on in a non-expressed gene in hESCs, which allows for the successful isolation of a specific pancreatic cell lineage.

We selected NGN3eGFP$^+$ cells from hESC progeny instructed to differentiate to endoderm with Activin-A and Wnt3, followed by differentiation towards endocrine pancreas. This protocol yielded sufficient number of cells to perform most studies, although optimization of the differentiation protocol would aid very much in increasing cell number. The NGN3eGFP$^+$ sorted cells could be further differentiated *in vitro* to endocrine cells when cultured with presence of IGF-II and NA, although no glucose-responsiveness could be detected. This is not unexpected as expression levels for *INS*, *GCG* and *SST* remained 10-100-fold lower than in human islets. As the number of NGN3eGFP$^+$ cells was low for *in vitro* differentiation, we could not assess other maturation protocols. We further demonstrated that pure NGN3eGFP$^+$ cells, when transplanted immediately after sorting under the kidney capsule of immunodeficient mice generate CHGA$^+$ progeny that co-stains mainly single-positive for INS, GCG or SST, while neither ductal nor exocrine pancreatic cells are identified. Although, no previous reports were published about transplantation conditions for NGN3$^+$ cells, we were able to detect some pancreatic endocrine cells. On the other hand many cells remained unidentified and were no longer NGN3eGFP$^+$. It

will be necessary to optimize the protocol to generate more NGN3eGFP$^+$ cells, to address the above questions. Lineage tracing studies in mouse demonstrated that Ngn3$^+$ cells from both embryonic as well as adult pancreas tissue act as the endocrine progenitors that gives rise to all endocrine cells and suggested that regeneration may recapitulate developmental pathways[37]. Xu *et al*. 2008 also demonstrated that Ngn3eGFP$^+$ cells isolated from regenerating pancreas in the adult mouse contains eGFP$^+$ low side scatter (eGFP/LSSC) cells that are similar to NGN3eGFP$^+$ cells from embryonic stage E13.5, further supporting the previous study[54]. Aside from the eGFP/LSSC population, another Ngn3$^+$ subpopulation (eGFP/HSSC), more granulated and believed to be more committed, was also detected and showed a comparable expression profile to the human ESC-derived NGN3eGFP$^+$ cells described here. Using differentiating hESCs, Kelly *et al.* 2011 demonstrated that CD142$^+$/PDX1$^+$/NKX6.1$^+$/CHGA$^-$ cells give rise to all three pancreatic cell types, and represent pancreatic endoderm[101]. Based on these murine and human studies, we believe, the NGN3eGFP$^+$ cells isolated in the current study, which are also PDX1$^+$ and NKX6.1$^+$, do not express CD142 or PTF1A, but express CHGA and mature *in vivo* to islet cells, are pancreatic endocrine progenitors. Other studies mention the longer half-life of eGFP compared to NGN3. In this report we were still able to detect NGN3 protein in all eGFP$^+$ cells by immunofluorescence. This does not exclude varying expression levels of NGN3 from cell to cell, i.e. that some cells could express higher levels of NGN3 compared to others[54, 135]. This is difficult to investigate, as the ESC-derived progeny are quite auto-fluorescent making discrimination between dim and bright GFP positive cells difficult. This could also explain why not all cells differentiated further into pancreatic endocrine cells. Nevertheless, our findings provide further insight in the development of pancreatic endocrine fate specification in human.

The NGN3eGFP-hESC lines will also be a useful tool for optimization of hESC differentiation protocols as it will allow high throughput screening for small molecules that increase the number of NGN3eGFP$^+$ cell fraction during differentiation[98, 99] as well as for molecules that may stimulate NGN3$^+$ cell differentiation towards β-cells. In addition, the purified NGN3eGFP$^+$ cell fraction should allow the identification of cell-surface markers that could then be used to detect NGN3$^+$ cells in fetal and postnatal tissues[37, 54]. Finally, it will also be of interest to test whether the NGN3eGFP-hESC line could be used to isolate glial, intestine or gut progenitors from hESC cultures directed towards the neuroectodermal or intestinal lineages.

In conclusion, we demonstrate that NGN3eGFP^{+} cells prospectively isolated from differentiating hESCs can develop further towards single-hormone positive cells *in vivo*, providing proof of principle for the purification of a human pancreatic endocrine progenitor. We also demonstrate that the NGN3eGFP knock-in add-on strategy is a reliable and powerful tool for lineage tracing purposes that could also be used in other areas of human stem cell research.

7 CONCLUSION AND FUTURE PERSPECTIVES

Advances in the understanding of pancreas development and the interesting possibilities that stem cells open for cell transplantation have set the pace for many investigators to start or proceed their research on DM type 1. As mentioned in the introduction, more and more people suffer from DM for which the only curative therapy is the transplantation of pancreata or islets of Langerhans[4]. Unfortunately, scarcity of donor tissue makes this option available to only few patients. Therefore, alternative cell sources for DM type 1 could relieve a part of this burden. Several routes have been pursued for this purpose ranging from regeneration, proliferation, differentiation, reprogramming and trans-determination. In this light, we choose to make use of the pluripotency and self-renewal potential of hESCs to better understand pancreatic development.

During this study, we were able to generate a powerful tool for lineage tracing purposes by combining ZFN technology with hESCs. We believe that the introduction of ZNFs and TALEN will push the field of generating reporter cell lines from hPSCs forward, as HR was hampered due to the low targeting efficiency. The reporter hESC cell lines for *NGN3* described here will open perspectives in tackling many developmental questions in the pancreas field. The main advantage of choosing *Ngn3* is that its role is well characterized in mouse endocrine pancreas development as the sole cell that gives rise to pancreatic endocrine islet cells[37, 38, 54, 135]. As *Pdx1* is more widely expressed this would isolate a multipotent progenitor instead of an endocrine progenitor[32]. Upon selecting NGN3eGFP$^+$ cells, we could identify many important endocrine pancreas genes to be expressed in these cells. We also demonstrated that NGN3eGFP$^+$ cells have the capacity to differentiate towards pancreatic endocrine cells *in vivo*, but not exocrine and ductal cells. Therefore, it will be interesting to identify a surface marker for the NGN3eGFP$^+$ cells that would allow isolation of pancreatic endocrine progenitors from human tissues. For this purpose, we have send samples for micro-array analysis, unfortunately due to time constraints; we could not add this data set to the thesis.

It will also be interesting to improve the protocol for generating more NGN3eGFP$^+$ cells from the reporter lines and to address their differentiation potential *in vitro* (using growth factors or

supporting feeders) or *in vivo* (using human pancreata). The advantage of using the prospective isolated NGN3eGFP$^+$ cells is that it will allow (semi) high-throughput screening for factors that enhance the generation of NGN3eGFP$^+$ cells. These studies should be able to move the current differentiation protocol towards a more chemically-defined medium for the induction of endocrine progenitor cells. Therefore, it would be interesting for example to look at the molecules identified by Melton's group, which appear to improve the commitment towards definitive endoderm (IDE1 and IDE2), or to pancreatic endoderm (indolactam V (ILV)[98, 99]. By improving the differentiation protocol, an increase in the amount of NGN3eGFP$^+$ cells should be possible. This will allow further *in vivo* studies for determining their differentiation capabilities towards e.g. intestine[127], gut[128] and glial cells[131] for which the NGN3-expressing cell is a common ancestor. It is also not yet clear, if there is a need of supporting cells for the *in vitro* differentiation of NGN3-expressing cells as it has been shown by Sander's group that endocrine cells cannot be terminally differentiated *in vitro*, due to aberrant histone modification marks. They showed this by comparing the epigenetic profile of *in vivo* matured with *in vitro* matured endocrine cells[115]. Therefore, it would be interesting to look *in vitro* at co-cultures which could improve their functionality.

Finally, if NGN3eGFP$^+$ cells could be directed towards functional β-cells, then this would generate a possible therapy for DM, although current transplantations in humans are performed with pancreatic islet cells and not with β-cells solely[4]. Looking at the self-renewal capacity of the sorted cells, as performed in a study by Sneddon *et al.* 2012, would be interesting as well. They showed that a specific cell type makes the difference between generating a self-renewable source or not[136]. In this way a novel alternative and expandable source would arise for transplantation and other research purposes where a large number of cells are needed.

Also, epigenetic questions using NGN3eGFP$^+$ cells, for the investigation of histone marks or methylation patterns, or looking at genuine pathways involved in pancreatic development, or the quest for specific cell-surface maker(s) can be addressed by comparing with other specifically isolated endodermal cells like the SOX17-expressing cells generated by Wang *et al.* 2011[100] or the CD142$^+$ cells from Kelly *et al.* 2011[101].

Making use of enriched (not purified) populations for research objectives is possible for comparing epigenetic profiles as shown by Xie *et al.* 2013, although the "contamination" with

other cell types is inevitable and can skew the data[115]. Using specific cell populations makes it possible to define pathways that regulate cell differentiation, survival, growth and other functions. Another way to use the cells is to look at specific cell-surface marker(s). Using these markers would help to validate NGN3-expressing cells in humans. Current studies rely on the use of rodents, of which results are not always applicable on human beings.

In conclusion, the need for a FACS-purified progenitor cell, like our system, is a must-have in analyzing and dissecting developmental mechanisms in the pancreas.

8 REFERENTIES

1. Dean L, McEntyre J. The genetic landscape of diabetes. National Center for Biotechnology Information (US), 2004.
2. Wohlrab J, Wohlrab D, Meiss F. Skin diseases in diabetes mellitus. J Dtsch Dermatol Ges 2007;5:37-53.
3. Yadav S, Parakh A. Insulin therapy. Indian pediatrics 2006;43:863-72.
4. Shapiro AM, Lakey JR, Ryan EA, Korbutt GS, Toth E, Warnock GL, Kneteman NM, Rajotte RV. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000;343:230-8.
5. Ryan EA, Lakey JR, Shapiro AM. Clinical results after islet transplantation. Journal of investigative medicine : the official publication of the American Federation for Clinical Research 2001;49:559-62.
6. Nussey S, Whitehead S. Endocrinology: An Integrated Approach. Oxford, 2001.
7. Gavin JR, Alberti KGMM, Davidson MB, DeFronzo RA, Drash A, Gabbe SG, Genuth S, Harris MI, Kahn R, Keen H, Knowler WC, Lebovitz H, Maclaren NK, Palmer JP, Raskin P, Rizza RA, Stern MP. Report of the expert committee on the diagnosis and classification of diabetes mellitus. Diabetes Care. Volume 26 Suppl 1, 2003:S5-20.
8. Edlund H. Pancreatic organogenesis--developmental mechanisms and implications for therapy. Nat Rev Genet 2002;3:524-32.
9. Donnelly R, Emslie-Smith AM, Gardner ID, Morris AD. ABC of arterial and venous disease: vascular complications of diabetes. Bmj 2000;320:1062-6.
10. Nussey SS, Whitehead SA. Endocrinology An integrated approach: BIOS Scientific Publishers Ltd, 2001.
11. Frykberg RG, Zgonis T, Armstrong DG, Driver VR, Giurini JM, Kravitz SR, Landsman AS, Lavery LA, Moore JC, Schuberth JM, Wukich DK, Andersen C, Vanore JV. DIABETIC FOOT DISORDERS: A CLINICAL PRACTICE GUIDELINE (2006 revision). J Foot Ankle Surg 2006;45:S1-S66.

12. Cheng AY, Fantus IG. Oral antihyperglycemic therapy for type 2 diabetes mellitus. Cmaj 2005;172:213-26.

13. Zaret KS. Genetic programming of liver and pancreas progenitors: lessons for stem-cell differentiation. Nature reviews. Genetics 2008;9:329-40.

14. Sander M, German MS. The beta cell transcription factors and development of the pancreas. J Mol Med 1997;75:327-40.

15. Jensen J. Gene regulatory factors in pancreatic development. Dev Dyn 2004;229:176-200.

16. Slack JM. Developmental biology of the pancreas. Development 1995;121:1569-80.

17. Gittes GK, Galante PE, Hanahan D, Rutter WJ, Debase HT. Lineage-specific morphogenesis in the developing pancreas: role of mesenchymal factors. Development 1996;122:439-47.

18. Kim SK, Hebrok M, Melton DA. Notochord to endoderm signaling is required for pancreas development. Development 1997;124:4243-52.

19. Kume S. The molecular basis and prospects in pancreatic development. Dev Growth Differ 2005;47:367-74.

20. Lee JC, Smith SB, Watada H, Lin J, Scheel D, Wang J, Mirmira RG, German MS. Regulation of the pancreatic pro-endocrine gene neurogenin3. Diabetes 2001;50:928-36.

21. Piper K, Brickwood S, Turnpenny LW, Cameron IT, Ball SG, Wilson DI, Hanley NA. Beta cell differentiation during early human pancreas development. The Journal of endocrinology 2004;181:11-23.

22. Polak M, Bouchareb-Banaei L, Scharfmann R, Czernichow P. Early pattern of differentiation in the human pancreas. Diabetes 2000;49:225-32.

23. Lyttle BM, Li J, Krishnamurthy M, Fellows F, Wheeler MB, Goodyer CG, Wang R. Transcription factor expression in the developing human fetal endocrine pancreas. Diabetologia 2008;51:1169-80.

24. Sarkar SA, Kobberup S, Wong R, Lopez AD, Quayum N, Still T, Kutchma A, Jensen JN, Gianani R, Beattie GM, Jensen J, Hayek A, Hutton JC. Global gene expression profiling

and histochemical analysis of the developing human fetal pancreas. Diabetologia 2008;51:285-97.

25. Castaing M, Peault B, Basmaciogullari A, Casal I, Czernichow P, Scharfmann R. Blood glucose normalization upon transplantation of human embryonic pancreas into beta-cell-deficient SCID mice. Diabetologia 2001;44:2066-76.

26. Castaing M, Duvillie B, Quemeneur E, Basmaciogullari A, Scharfmann R. Ex vivo analysis of acinar and endocrine cell development in the human embryonic pancreas. Developmental dynamics : an official publication of the American Association of Anatomists 2005;234:339-45.

27. Sugiyama T, Rodriguez RT, McLean GW, Kim SK. Conserved markers of fetal pancreatic epithelium permit prospective isolation of islet progenitor cells by FACS. Proceedings of the National Academy of Sciences of the United States of America 2007;104:175-80.

28. Jeon J, Correa-Medina M, Ricordi C, Edlund H, Diez JA. Endocrine cell clustering during human pancreas development. The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society 2009;57:811-24.

29. Kim SK, MacDonald RJ. Signaling and transcriptional control of pancreatic organogenesis. Curr Opin Genet Dev 2002;12:540-7.

30. Bonner-Weir S, Orci L. New perspectives on the microvasculature of the islets of Langerhans in the rat. Diabetes 1982;31:883-9.

31. Stoffers DA, Thomas MK, Habener JF. Homeodomain protein IDX-1: a master regulator of pancreas development and insulin gene expression. Trends in endocrinology and metabolism: TEM 1997;8:145-51.

32. Ohlsson H, Karlsson K, Edlund T. IPF1, a homeodomain-containing transactivator of the insulin gene. The EMBO journal 1993;12:4251-9.

33. Kawaguchi Y, Cooper B, Gannon M, Ray M, MacDonald RJ, Wright CV. The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. Nat Genet 2002;32:128-34.

34. Seymour PA, Freude KK, Tran MN, Mayes EE, Jensen J, Kist R, Scherer G, Sander M. SOX9 is required for maintenance of the pancreatic progenitor cell pool. Proceedings of the National Academy of Sciences of the United States of America 2007;104:1865-70.

35. Jensen J, Pedersen EE, Galante P, Hald J, Heller RS, Ishibashi M, Kageyama R, Guillemot F, Serup P, Madsen OD. Control of endodermal endocrine development by Hes-1. Nat Genet 2000;24:36-44.

36. Apelqvist A, Li H, Sommer L, Beatus P, Anderson DJ, Honjo T, Hrabe de Angelis M, Lendahl U, Edlund H. Notch signalling controls pancreatic cell differentiation. Nature 1999;400:877-81.

37. Gu G, Dubauskaite J, Melton DA. Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development 2002;129:2447-57.

38. Gradwohl G, Dierich A, LeMeur M, Guillemot F. neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc Natl Acad Sci U S A 2000;97:1607-11.

39. Sosa-Pineda B, Chowdhury K, Torres M, Oliver G, Gruss P. The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas. Nature 1997;386:399-402.

40. Collombat P, Mansouri A, Hecksher-Sorensen J, Serup P, Krull J, Gradwohl G, Gruss P. Opposing actions of Arx and Pax4 in endocrine pancreas development. Genes & development 2003;17:2591-603.

41. Sussel L, Kalamaras J, Hartigan-O'Connor DJ, Meneses JJ, Pedersen RA, Rubenstein JL, German MS. Mice lacking the homeodomain transcription factor Nkx2.2 have diabetes due to arrested differentiation of pancreatic beta cells. Development 1998;125:2213-21.

42. Sander M, Sussel L, Conners J, Scheel D, Kalamaras J, Dela Cruz F, Schwitzgebel V, Hayes-Jordan A, German M. Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of beta-cell formation in the pancreas. Development 2000;127:5533-40.

43. Duncan AW, Dorrell C, Grompe M. Stem cells and liver regeneration. Gastroenterology 2009;137:466-81.

44. Espanol-Suner R, Lemaigre FP, Leclercq IA. Reply. Gastroenterology 2013;145:255-6.

45. Dor Y, Brown J, Martinez OI, Melton DA. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 2004;429:41-6.

46. Nir T, Melton DA, Dor Y. Recovery from diabetes in mice by beta cell regeneration. J Clin Invest 2007;117:2553-61.

47. Teta M, Rankin MM, Long SY, Stein GM, Kushner JA. Growth and regeneration of adult beta cells does not involve specialized progenitors. Developmental cell 2007;12:817-26.

48. Gershengorn MC, Hardikar AA, Wei C, Geras-Raaka E, Marcus-Samuels B, Raaka BM. Epithelial-to-mesenchymal transition generates proliferative human islet precursor cells. Science 2004;306:2261-4.

49. Meier JJ, Butler AE, Saisho Y, Monchamp T, Galasso R, Bhushan A, Rizza RA, Butler PC. Beta-cell replication is the primary mechanism subserving the postnatal expansion of beta-cell mass in humans. Diabetes 2008;57:1584-94.

50. Reers C, Erbel S, Esposito I, Schmied B, Buchler MW, Nawroth PP, Ritzel RA. Impaired islet turnover in human donor pancreata with aging. European journal of endocrinology / European Federation of Endocrine Societies 2009;160:185-91.

51. Butler AE, Cao-Minh L, Galasso R, Rizza RA, Corradin A, Cobelli C, Butler PC. Adaptive changes in pancreatic beta cell fractional area and beta cell turnover in human pregnancy. Diabetologia 2010;53:2167-76.

52. Karnik SK, Chen H, McLean GW, Heit JJ, Gu X, Zhang AY, Fontaine M, Yen MH, Kim SK. Menin controls growth of pancreatic beta-cells in pregnant mice and promotes gestational diabetes mellitus. Science 2007;318:806-9.

53. Lee CS, De Leon DD, Kaestner KH, Stoffers DA. Regeneration of pancreatic islets after partial pancreatectomy in mice does not involve the reactivation of neurogenin-3. Diabetes 2006;55:269-72.

54. Xu X, D'Hoker J, Stange G, Bonne S, De Leu N, Xiao X, Van de Casteele M, Mellitzer G, Ling Z, Pipeleers D, Bouwens L, Scharfmann R, Gradwohl G, Heimberg H. Beta cells

can be generated from endogenous progenitors in injured adult mouse pancreas. Cell 2008;132:197-207.

55. Collombat P, Xu X, Ravassard P, Sosa-Pineda B, Dussaud S, Billestrup N, Madsen OD, Serup P, Heimberg H, Mansouri A. The ectopic expression of Pax4 in the mouse pancreas converts progenitor cells into alpha and subsequently beta cells. Cell 2009;138:449-62.

56. Gao R, Ustinov J, Pulkkinen MA, Lundin K, Korsgren O, Otonkoski T. Characterization of endocrine progenitor cells and critical factors for their differentiation in human adult pancreatic cell culture. Diabetes 2003;52:2007-15.

57. Bonner-Weir S, Taneja M, Weir GC, Tatarkiewicz K, Song KH, Sharma A, O'Neil JJ. In vitro cultivation of human islets from expanded ductal tissue. Proceedings of the National Academy of Sciences of the United States of America 2000;97:7999-8004.

58. Ferber S, Halkin A, Cohen H, Ber I, Einav Y, Goldberg I, Barshack I, Seijffers R, Kopolovic J, Kaiser N, Karasik A. Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. Nature medicine 2000;6:568-72.

59. Kojima H, Fujimiya M, Matsumura K, Younan P, Imaeda H, Maeda M, Chan L. NeuroD-betacellulin gene therapy induces islet neogenesis in the liver and reverses diabetes in mice. Nature medicine 2003;9:596-603.

60. Yechoor V, Liu V, Paul A, Lee J, Buras E, Ozer K, Samson S, Chan L. Gene therapy with neurogenin 3 and betacellulin reverses major metabolic problems in insulin-deficient diabetic mice. Endocrinology 2009;150:4863-73.

61. Zheng YW, Taniguchi H. Diversity of hepatic stem cells in the fetal and adult liver. Seminars in liver disease 2003;23:337-48.

62. Zalzman M, Anker-Kitai L, Efrat S. Differentiation of human liver-derived, insulin-producing cells toward the beta-cell phenotype. Diabetes 2005;54:2568-75.

63. Zalzman M, Gupta S, Giri RK, Berkovich I, Sappal BS, Karnieli O, Zern MA, Fleischer N, Efrat S. Reversal of hyperglycemia in mice by using human expandable insulin-

producing cells differentiated from fetal liver progenitor cells. Proceedings of the National Academy of Sciences of the United States of America 2003;100:7253-8.

64. Sapir T, Shternhall K, Meivar-Levy I, Blumenfeld T, Cohen H, Skutelsky E, Eventov-Friedman S, Barshack I, Goldberg I, Pri-Chen S, Ben-Dor L, Polak-Charcon S, Karasik A, Shimon I, Mor E, Ferber S. Cell-replacement therapy for diabetes: Generating functional insulin-producing tissue from adult human liver cells. Proceedings of the National Academy of Sciences of the United States of America 2005;102:7964-9.

65. Zhou Q, Brown J, Kanarek A, Rajagopal J, Melton DA. In vivo reprogramming of adult pancreatic exocrine cells to beta-cells. Nature 2008;455:627-32.

66. Zhou Q, Law AC, Rajagopal J, Anderson WJ, Gray PA, Melton DA. A multipotent progenitor domain guides pancreatic organogenesis. Developmental cell 2007;13:103-14.

67. Ianus A, Holz GG, Theise ND, Hussain MA. In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. The Journal of clinical investigation 2003;111:843-50.

68. Lechner A, Yang YG, Blacken RA, Wang L, Nolan AL, Habener JF. No evidence for significant transdifferentiation of bone marrow into pancreatic beta-cells in vivo. Diabetes 2004;53:616-23.

69. Butler AE, Huang A, Rao PN, Bhushan A, Hogan WJ, Rizza RA, Butler PC. Hematopoietic stem cells derived from adult donors are not a source of pancreatic beta-cells in adult nondiabetic humans. Diabetes 2007;56:1810-6.

70. Friedenstein AJ, Deriglasova UF, Kulagina NN, Panasuk AF, Rudakowa SF, Luria EA, Ruadkow IA. Precursors for fibroblasts in different populations of hematopoietic cells as detected by the in vitro colony assay method. Experimental hematology 1974;2:83-92.

71. Caplan AI. Mesenchymal stem cells. Journal of orthopaedic research : official publication of the Orthopaedic Research Society 1991;9:641-50.

72. Prockop DJ. Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 1997;276:71-4.

73. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR. Multilineage potential of adult human mesenchymal stem cells. Science 1999;284:143-7.

74. Lee RH, Seo MJ, Reger RL, Spees JL, Pulin AA, Olson SD, Prockop DJ. Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice. Proceedings of the National Academy of Sciences of the United States of America 2006;103:17438-43.

75. Sordi V, Melzi R, Mercalli A, Formicola R, Doglioni C, Tiboni F, Ferrari G, Nano R, Chwalek K, Lammert E, Bonifacio E, Borg D, Piemonti L. Mesenchymal cells appearing in pancreatic tissue culture are bone marrow-derived stem cells with the capacity to improve transplanted islet function. Stem Cells 2010;28:140-51.

76. Ho JH, Tseng TC, Ma WH, Ong WK, Chen YF, Chen MH, Lin MW, Hong CY, Lee OK. Multiple intravenous transplantations of mesenchymal stem cells effectively restore long-term blood glucose homeostasis by hepatic engraftment and beta-cell differentiation in streptozocin-induced diabetic mice. Cell transplantation 2012;21:997-1009.

77. Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM. Embryonic stem cell lines derived from human blastocysts. Science 1998;282:1145-7.

78. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 2007;131:861-72.

79. Robinton DA, Daley GQ. The promise of induced pluripotent stem cells in research and therapy. Nature 2012;481:295-305.

80. Mayshar Y, Ben-David U, Lavon N, Biancotti JC, Yakir B, Clark AT, Plath K, Lowry WE, Benvenisty N. Identification and classification of chromosomal aberrations in human induced pluripotent stem cells. Cell Stem Cell 2010;7:521-31.

81. Taapken SM, Nisler BS, Newton MA, Sampsell-Barron TL, Leonhard KA, McIntire EM, Montgomery KD. Karotypic abnormalities in human induced pluripotent stem cells and embryonic stem cells. Nature biotechnology 2011;29:313-4.

82. Nazor KL, Altun G, Lynch C, Tran H, Harness JV, Slavin I, Garitaonandia I, Muller FJ, Wang YC, Boscolo FS, Fakunle E, Dumevska B, Lee S, Park HS, Olee T, D'Lima DD, Semechkin R, Parast MM, Galat V, Laslett AL, Schmidt U, Keirstead HS, Loring JF, Laurent LC. Recurrent variations in DNA methylation in human pluripotent stem cells and their differentiated derivatives. Cell Stem Cell 2012;10:620-34.

83. Guenther MG, Frampton GM, Soldner F, Hockemeyer D, Mitalipova M, Jaenisch R, Young RA. Chromatin structure and gene expression programs of human embryonic and induced pluripotent stem cells. Cell Stem Cell 2010;7:249-57.

84. Araki R, Uda M, Hoki Y, Sunayama M, Nakamura M, Ando S, Sugiura M, Ideno H, Shimada A, Nifuji A, Abe M. Negligible immunogenicity of terminally differentiated cells derived from induced pluripotent or embryonic stem cells. Nature 2013;494:100-4.

85. Maherali N, Ahfeldt T, Rigamonti A, Utikal J, Cowan C, Hochedlinger K. A high-efficiency system for the generation and study of human induced pluripotent stem cells. Cell Stem Cell 2008;3:340-5.

86. Aoi T, Yae K, Nakagawa M, Ichisaka T, Okita K, Takahashi K, Chiba T, Yamanaka S. Generation of pluripotent stem cells from adult mouse liver and stomach cells. Science 2008;321:699-702.

87. Miura K, Okada Y, Aoi T, Okada A, Takahashi K, Okita K, Nakagawa M, Koyanagi M, Tanabe K, Ohnuki M, Ogawa D, Ikeda E, Okano H, Yamanaka S. Variation in the safety of induced pluripotent stem cell lines. Nature biotechnology 2009;27:743-5.

88. Kim K, Doi A, Wen B, Ng K, Zhao R, Cahan P, Kim J, Aryee MJ, Ji H, Ehrlich LI, Yabuuchi A, Takeuchi A, Cunniff KC, Hongguang H, McKinney-Freeman S, Naveiras O, Yoon TJ, Irizarry RA, Jung N, Seita J, Hanna J, Murakami P, Jaenisch R, Weissleder R, Orkin SH, Weissman IL, Feinberg AP, Daley GQ. Epigenetic memory in induced pluripotent stem cells. Nature 2010;467:285-90.

89. Polo JM, Liu S, Figueroa ME, Kulalert W, Eminli S, Tan KY, Apostolou E, Stadtfeld M, Li Y, Shioda T, Natesan S, Wagers AJ, Melnick A, Evans T, Hochedlinger K. Cell type of origin influences the molecular and functional properties of mouse induced pluripotent stem cells. Nature biotechnology 2010;28:848-55.

90. Bar-Nur O, Russ HA, Efrat S, Benvenisty N. Epigenetic memory and preferential lineage-specific differentiation in induced pluripotent stem cells derived from human pancreatic islet beta cells. Cell Stem Cell 2011;9:17-23.

91. Kubo A, Shinozaki K, Shannon JM, Kouskoff V, Kennedy M, Woo S, Fehling HJ, Keller G. Development of definitive endoderm from embryonic stem cells in culture. Development 2004;131:1651-62.

92. D'Amour KA, Bang AG, Eliazer S, Kelly OG, Agulnick AD, Smart NG, Moorman MA, Kroon E, Carpenter MK, Baetge EE. Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells. Nat Biotechnol 2006;24:1392-401.

93. Hebrok M, Kim SK, St Jacques B, McMahon AP, Melton DA. Regulation of pancreas development by hedgehog signaling. Development 2000;127:4905-13.

94. Mfopou JK, Chen B, Sui L, Sermon K, Bouwens L. Recent advances and prospects in the differentiation of pancreatic cells from human embryonic stem cells. Diabetes 2010;59:2094-101.

95. Kroon E, Martinson LA, Kadoya K, Bang AG, Kelly OG, Eliazer S, Young H, Richardson M, Smart NG, Cunningham J, Agulnick AD, D'Amour KA, Carpenter MK, Baetge EE. Pancreatic endoderm derived from human embryonic stem cells generates glucose-responsive insulin-secreting cells in vivo. Nat Biotechnol 2008;26:443-52.

96. Matveyenko AV, Georgia S, Bhushan A, Butler PC. Inconsistent formation and nonfunction of insulin-positive cells from pancreatic endoderm derived from human embryonic stem cells in athymic nude rats. Am J Physiol Endocrinol Metab 2010;299:E713-20.

97. Alipio Z, Liao W, Roemer EJ, Waner M, Fink LM, Ward DC, Ma Y. Reversal of hyperglycemia in diabetic mouse models using induced-pluripotent stem (iPS)-derived pancreatic beta-like cells. Proceedings of the National Academy of Sciences of the United States of America 2010;107:13426-31.

98. Chen S, Borowiak M, Fox JL, Maehr R, Osafune K, Davidow L, Lam K, Peng LF, Schreiber SL, Rubin LL, Melton D. A small molecule that directs differentiation of human ESCs into the pancreatic lineage. Nat Chem Biol 2009;5:258-65.

99. Borowiak M, Maehr R, Chen S, Chen AE, Tang W, Fox JL, Schreiber SL, Melton DA. Small molecules efficiently direct endodermal differentiation of mouse and human embryonic stem cells. Cell Stem Cell 2009;4:348-58.

100. Wang P, Rodriguez RT, Wang J, Ghodasara A, Kim SK. Targeting SOX17 in human embryonic stem cells creates unique strategies for isolating and analyzing developing endoderm. Cell Stem Cell 2011;8:335-46.

101. Kelly OG, Chan MY, Martinson LA, Kadoya K, Ostertag TM, Ross KG, Richardson M, Carpenter MK, D'Amour KA, Kroon E, Moorman M, Baetge EE, Bang AG. Cell-surface markers for the isolation of pancreatic cell types derived from human embryonic stem cells. Nat Biotechnol 2011;29:750-6.

102. Pagliuca FW, Melton DA. How to make a functional beta-cell. Development 2013;140:2472-83.

103. Hentze H, Graichen R, Colman A. Cell therapy and the safety of embryonic stem cell-derived grafts. Trends Biotechnol 2007;25:24-32.

104. Davis RP, Ng ES, Costa M, Mossman AK, Sourris K, Elefanty AG, Stanley EG. Targeting a GFP reporter gene to the MIXL1 locus of human embryonic stem cells identifies human primitive streak-like cells and enables isolation of primitive hematopoietic precursors. Blood 2008;111:1876-84.

105. Zwaka TP, Thomson JA. Homologous recombination in human embryonic stem cells. Nat Biotechnol 2003;21:319-21.

106. Hockemeyer D, Soldner F, Beard C, Gao Q, Mitalipova M, DeKelver RC, Katibah GE, Amora R, Boydston EA, Zeitler B, Meng X, Miller JC, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Jaenisch R. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. Nat Biotechnol 2009;27:851-7.

107. Song H, Chung SK, Xu Y. Modeling disease in human ESCs using an efficient BAC-based homologous recombination system. Cell stem cell 2010;6:80-9.

108. Hockemeyer D, Soldner F, Beard C, Gao Q, Mitalipova M, DeKelver RC, Katibah GE, Amora R, Boydston EA, Zeitler B, Meng X, Miller JC, Zhang L, Rebar EJ, Gregory PD,

Urnov FD, Jaenisch R. Efficient targeting of expressed and silent genes in human ESCs and iPSCs using zinc-finger nucleases. Nature biotechnology 2009;27:851-7.

109. Hockemeyer D, Wang H, Kiani S, Lai CS, Gao Q, Cassady JP, Cost GJ, Zhang L, Santiago Y, Miller JC, Zeitler B, Cherone JM, Meng X, Hinkley SJ, Rebar EJ, Gregory PD, Urnov FD, Jaenisch R. Genetic engineering of human pluripotent cells using TALE nucleases. Nature biotechnology 2011;29:731-4.

110. Mali P, Yang L, Esvelt KM, Aach J, Guell M, DiCarlo JE, Norville JE, Church GM. RNA-guided human genome engineering via Cas9. Science 2013;339:823-6.

111. Ding Q, Lee YK, Schaefer EA, Peters DT, Veres A, Kim K, Kuperwasser N, Motola DL, Meissner TB, Hendriks WT, Trevisan M, Gupta RM, Moisan A, Banks E, Friesen M, Schinzel RT, Xia F, Tang A, Xia Y, Figueroa E, Wann A, Ahfeldt T, Daheron L, Zhang F, Rubin LL, Peng LF, Chung RT, Musunuru K, Cowan CA. A TALEN genome-editing system for generating human stem cell-based disease models. Cell Stem Cell 2013;12:238-51.

112. Elliott DA, Braam SR, Koutsis K, Ng ES, Jenny R, Lagerqvist EL, Biben C, Hatzistavrou T, Hirst CE, Yu QC, Skelton RJ, Ward-van Oostwaard D, Lim SM, Khammy O, Li X, Hawes SM, Davis RP, Goulburn AL, Passier R, Prall OW, Haynes JM, Pouton CW, Kaye DM, Mummery CL, Elefanty AG, Stanley EG. NKX2-5(eGFP/w) hESCs for isolation of human cardiac progenitors and cardiomyocytes. Nature methods 2011;8:1037-40.

113. Nostro MC, Sarangi F, Ogawa S, Holtzinger A, Corneo B, Li X, Micallef SJ, Park IH, Basford C, Wheeler MB, Daley GQ, Elefanty AG, Stanley EG, Keller G. Stage-specific signaling through TGFbeta family members and WNT regulates patterning and pancreatic specification of human pluripotent stem cells. Development 2011;138:861-71.

114. Basford CL, Prentice KJ, Hardy AB, Sarangi F, Micallef SJ, Li X, Guo Q, Elefanty AG, Stanley EG, Keller G, Allister EM, Nostro MC, Wheeler MB. The functional and molecular characterisation of human embryonic stem cell-derived insulin-positive cells compared with adult pancreatic beta cells. Diabetologia 2012;55:358-71.

115. Xie R, Everett LJ, Lim HW, Patel NA, Schug J, Kroon E, Kelly OG, Wang A, D'Amour KA, Robins AJ, Won KJ, Kaestner KH, Sander M. Dynamic Chromatin Remodeling Mediated by Polycomb Proteins Orchestrates Pancreatic Differentiation of Human Embryonic Stem Cells. Cell Stem Cell 2013.

116. Pyle AD, Lock LF, Donovan PJ. Neurotrophins mediate human embryonic stem cell survival. Nat Biotechnol 2006;24:344-50.

117. Hohenstein KA, Pyle AD, Chern JY, Lock LF, Donovan PJ. Nucleofection mediates high-efficiency stable gene knockdown and transgene expression in human embryonic stem cells. Stem Cells 2008;26:1436-43.

118. Watanabe K, Ueno M, Kamiya D, Nishiyama A, Matsumura M, Wataya T, Takahashi JB, Nishikawa S, Muguruma K, Sasai Y. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. Nat Biotechnol 2007;25:681-6.

119. Rezania A, Bruin JE, Riedel MJ, Mojibian M, Asadi A, Xu J, Gauvin R, Narayan K, Karanu F, O'Neil JJ, Ao Z, Warnock GL, Kieffer TJ. Maturation of Human Embryonic Stem Cell-Derived Pancreatic Progenitors into Functional Islets Capable of Treating Pre-existing Diabetes in Mice. Diabetes 2012.

120. Jiang W, Shi Y, Zhao D, Chen S, Yong J, Zhang J, Qing T, Sun X, Zhang P, Ding M, Li D, Deng H. In vitro derivation of functional insulin-producing cells from human embryonic stem cells. Cell research 2007;17:333-44.

121. Shim JH, Kim SE, Woo DH, Kim SK, Oh CH, McKay R, Kim JH. Directed differentiation of human embryonic stem cells towards a pancreatic cell fate. Diabetologia 2007;50:1228-38.

122. Apelqvist A, Ahlgren U, Edlund H. Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas. Curr Biol 1997;7:801-4.

123. Kim SK, Hebrok M, Li E, Oh SP, Schrewe H, Harmon EB, Lee JS, Melton DA. Activin receptor patterning of foregut organogenesis. Genes Dev 2000;14:1866-71.

124. Mfopou JK, Chen B, Mateizel I, Sermon K, Bouwens L. Noggin, retinoids, and fibroblast growth factor regulate hepatic or pancreatic fate of human embryonic stem cells. Gastroenterology 2010;138:2233-45, 2245 e1-14.

125. Otonkoski T, Beattie GM, Mally MI, Ricordi C, Hayek A. Nicotinamide is a potent inducer of endocrine differentiation in cultured human fetal pancreatic cells. The Journal of clinical investigation 1993;92:1459-66.

126. Jiang J, Au M, Lu K, Eshpeter A, Korbutt G, Fisk G, Majumdar AS. Generation of insulin-producing islet-like clusters from human embryonic stem cells. Stem Cells 2007;25:1940-53.

127. Osafune K, Caron L, Borowiak M, Martinez RJ, Fitz-Gerald CS, Sato Y, Cowan CA, Chien KR, Melton DA. Marked differences in differentiation propensity among human embryonic stem cell lines. Nature biotechnology 2008;26:313-5.

128. Scarff KL, Ung KS, Sun J, Bird PI. A retained selection cassette increases reporter gene expression without affecting tissue distribution in SPI3 knockout/GFP knock-in mice. Genesis 2003;36:149-57.

129. Burlison JS, Long Q, Fujitani Y, Wright CV, Magnuson MA. Pdx-1 and Ptf1a concurrently determine fate specification of pancreatic multipotent progenitor cells. Developmental biology 2008;316:74-86.

130. Jacquemin P, Durviaux SM, Jensen J, Godfraind C, Gradwohl G, Guillemot F, Madsen OD, Carmeliet P, Dewerchin M, Collen D, Rousseau GG, Lemaigre FP. Transcription factor hepatocyte nuclear factor 6 regulates pancreatic endocrine cell differentiation and controls expression of the proendocrine gene ngn3. Molecular and cellular biology 2000;20:4445-54.

131. Lee J, Wu Y, Qi Y, Xue H, Liu Y, Scheel D, German M, Qiu M, Guillemot F, Rao M, Gradwohl G. Neurogenin3 participates in gliogenesis in the developing vertebrate spinal cord. Developmental biology 2003;253:84-98.

132. Lee CS, Perreault N, Brestelli JE, Kaestner KH. Neurogenin 3 is essential for the proper specification of gastric enteroendocrine cells and the maintenance of gastric epithelial cell identity. Genes & development 2002;16:1488-97.

133. Jenny M, Uhl C, Roche C, Duluc I, Guillermin V, Guillemot F, Jensen J, Kedinger M, Gradwohl G. Neurogenin3 is differentially required for endocrine cell fate specification in the intestinal and gastric epithelium. The EMBO journal 2002;21:6338-47.

134. Niwa H, Miyazaki J, Smith AG. Quantitative expression of Oct-3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet 2000;24:372-6.

135. Mellitzer G, Martin M, Sidhoum-Jenny M, Orvain C, Barths J, Seymour PA, Sander M, Gradwohl G. Pancreatic islet progenitor cells in neurogenin 3-yellow fluorescent protein knock-add-on mice. Molecular endocrinology 2004;18:2765-76.

136. Sneddon JB, Borowiak M, Melton DA. Self-renewal of embryonic-stem-cell-derived progenitors by organ-matched mesenchyme. Nature 2012;491:765-8.

9 APPENDIX

Sequence of the donor construct

The donor construct (flanked by BamHI and NsiI) was cloned into the pCR2.1 vector (Life Technologies, KNM2040) using its multiple cloning site which contains BamHI and NsiI restriction site.

BamHI – 5' homology arm – T2A – eGFP – P2A - Puro^{R} – polyA – loxP - EF1α - – Hyg/TK – polyA - loxP – 3' homology arm -NsiI

GGATCCgttaacCTGACCCAGCGGGGGCTGGGAGGATGGCTGTTTTTGTTTTTTCCCACCTAGCCTCGGAA
TCGCGGACTGCGCCCAGTGACGGACTCAAACTTACCCTTCCCTCTGACCCCGCCGTAGGATGACGCCT
CAACCCTCGGGTGCGCCCACTGTCCAAGTGACCCGTGAGACGGAGCGGTCCTTCCCCAGAGCCTCGGA
AGACGAAGTGACCTGCCCCACGTCCGCCCCGCCCAGCCCCACTCGCACACGGGGGAACTGCGCAGAG
GCGGAAGAGGGAGGCTGCCGAGGGGCCCCGAGGAAGCTCCGGGCACGGCGCGGGGGACGCAGCCGG
CCTAAGAGCGAGTTGGCACTGAGCAAGCAGCGACGGAGTCGGCGAAAGAAGGCCAACGACCGCGAG
CGCAATCGAATGCACAACCTCAACTCGGCACTGGACGCCCTGCGCGGTGTCCTGCCCACCTTCCCAGA
CGACGCGAAGCTCACCAAGATCGAGACGCTGCGCTTCGCCCACAACTACATCTGGGCGCTGACTCAAA
CGCTGCGCATAGCGGACCACAGCTTGTACGCGCTGGAGCCGCCGGCGCCGCACTGCGGGGAGCTGGG
CAGCCCAGGCGGTTCCCCCGGGGACTGGGGGTCCCTCTACTCCCCAGTCTCCCAGGCTGGCAGCCTGA
GTCCCGCCGCGTCGCTGGAGGAGCGACCCGGGCTGCTGGGTGCAACATTCTCAGCTTGTTTGAGTCCG
GGAAGCCTCGCTTTCTCAGATTTTCTGgctagcGGAGGTGGCAGCGGTGGCGGTGAGGGCAGAGGAAGTC
TTCTAACATGCGGTGACGTGGAGGAGAATCCCGGCCCTcctAGGATGGTGAGCAAGGGCGAGGAGCTGTTCA
CCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAG
GGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCT
CGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGT
CCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAG
GTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGAGGACGGCAACATCCT
GGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACGGCATCAAGG
TGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATC
GGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAA
GCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGggtggt
ggctcgggggaggagcgacaaactttagcttgctgaagcaagctggtgacgttgaggagaatcccggaccaggattc**acgcgtATG**accgagtacaagc
ccacggtgcgcctcgccacccgcgacgacgtccccgggccgtacgcaccctcgccgccgcgttcgccgactacccc
gccacgcgccacaccgtcgacccggaccgccacatcgagcgggtcaccgagctgcaagaactcttcctcacgcgcgt
cgggctcgacatcggcaaggtgtgggtcgcggacgacggcgccgcggtggcggtctggaccacgccggagagcgtcg
aagcgggggcggtgttcgccgagatcggcccgcgcatggccgagttgagcggttcccggctggccgcgcagcaacag

atggaaggcctcctggcgccgcaccggcccaaggagcccgcgtggttcctggccaccgtcggcgtctcgcccgacca
ccagggcaagggtctgggcagcgccgtcgtgctccccggagtggaggcggccgagcgcgccggggtgcccgccttcc
tggagacctccgcgccccgcaacctccccttctacgagcggctcggcttcaccgtcaccgccgacgtcgaggtgccc
gaaggaccgcgcacctggtgcatgacccgcaagcccggtgcc**TGA**cgcccgccccacgacccgcagcgcccgaccga
aaggagcgcacgaccccatggctccgaccgaagccacccggggcggccccgccgaccccgcacccgcccccgaggcc
caccgactctagagggcccgtttaaacccgctgatcagcctcgactgtgccttctagttgccagccatctgttgttt
gcccctcccccgtgccttccttgaccctggaaggtgccactcccactgtcctttcctaataaaatgaggaaattgca
tcgcattgtctgagtaggtgtcattctattctgggggggtggggtggggcaggacagcaagggggaggattgggaaga
caatagca**ttcgaa**ataacttcgtatagtatacattatacgaagttatTCTAGAGGCTCCGGTGCCCGTCAGTGGGC
AGAGCGCACATCGCCCACAGTCCCCGAGAAGTTGGGGGGAGGGGTCGGCAATTGAACCGGTGCCTAGAGAAGGTGGC
GCGGGGTAAACTGGGAAAGTGATGTCGTGTACTGGCTCCGCCTTTTTCCCGAGGGTGGGGGAGAACCGTATATAAGT
GCAGTAGTCGCCGTGAACGTT**CTTTTTCGCAACGGGTTTGCCGCCAGAACACAG**GTAAGTGCCGTGTGTGGTTCCCG
CGGGCCTGGCCTCTTTACGGGTTATGGCCCTTGCGTGCCTTGAATTACTTCCACCTGGCTGCAGTACGTGATTCTTG
ATCCCGAGCTTCGGGTTGGAAGTGGGTGGGAGAGTTCGAGGCCTTGCGCTTAAGGAGCCCCTTCGCCTCGTGCTTGA
GTTGAGGCCTGGCCTGGGCGCTGGGGCCGCCGCGTGCGAATCTGGTGGCACCTTCGCGCCTGTCTCGCTGCTTTCGA
TAAGTCTCTAGCCATTTAAAATTTTTGATGACCTGCTGCGACGCTTTTTTTCTGGCAAGATAGTCTTGTAAATGCGG
GCCAAGATCTGCACACTGGTATTTCGGTTTTTGGGGCCGCGGGCGGCGACGGGGCCCGTGCGTCCCAGCGCACATGT
TCGGCGAGGCGGGGCCTGCGAGCGCGGCCACCGAGAATCGGACGGGGGTAGTCTCAAGCTGGCCGGCCTGCTCTGGT
GCCTGGCCTCGCGCCGCCGTGTATCGCCCCGCCCTGGGCGGCAAGGCTGGCCCGGTCGGCACCAGTTGCGTGAGCGG
AAAGATGGCCGCTTCCCGGCCCTGCTGCAGGGAGCTCAAAATGGAGGACGCGGCGCTCGGGAGAGCGGGCGGGTGAG
TCACCCACACAAAGGAAAAGGGCCTTTCCGTCCTCAGCCGTCGCTTCATGTGACTCCACGGAGTACCGGGCGCCGTC
CAGGCACCTCGATTAGTTCTCGAGCTTTTGGAGTACGTCGTCTTTAGGTTGGGGGGAGGGGTTTTATGCGATGGAGT
TTCCCCACACTGAGTGGGTGGAGACTGAAGTTAGGCCAGCTTGGCACTTGATGTAATTCTCCTTGGAATTTGCCCTT
TTTGAGTTTGGATCTTGGTTCATTCTCAAGCCTCAGACAGTGGTTCAAAGTTTTTTTCTTCCATTTCAG**GTGTCGTG**
AG**GAATTC***GCCACC***ATG**aaaaagcctgaactcaccgcgacgtctgtcgagaagtttctgatcgaaaagttcgacagc
gtctccgacctgatgcagctctcggagggcgaagaatctcgtgctttcagcttcgatgtaggagggcgtggatatgt
cctgcgggtaaatagctgcgccgatggtttctacaaagatcgttatgtttatcggcactttgcatcggccgcgctcc
cgattccggaagtgcttgacattggggaattcagcgagagcctgacctattgcatctcccgccgtgcacagggtgtc
acgttgcaagacctgcctgaaaccgaactgcccgctgttctgcagccggtcgcggaggccatggatgcgatcgctgc
ggccgatcttagccagacgagcgggttcggcccattcggaccgcaaggaatcggtcaatacactacatggcgtgatt
tcatatgcgcgattgctgatccccatgtgtatcactggcaaactgtgatggacgacaccgtcagtgcgtccgtcgcg
caggctctcgatgagctgatgctttgggccgaggactgccccgaagtccggcacctcgtgcacgcggatttcggctc
caacaatgtcctgacggacaatggccgcataacagcggtcattgactggagcgaggcgatgttcggggattcccaat
acgaggtcgccaacatcttcttctggaggccgtggttggcttgtatggagcagcagacgcgctacttcgagcggagg
catccggagcttgcaggatcgccgcggctccgggcgtatatgctccgcattggtcttgaccaactctatcagagctt
ggttgacggcaatttcgatgatgcagcttgggcgcagggtcgatgcgacgcaatcgtccgatccggagccgggactg
tcgggcgtacacaaatcgcccgcagaagcgcggccgtctggaccgatggctgtgtagaagtcgcgtctgcgttcgac
caggctgcgcgttctcgcggccatagcaaccgacgtacggcgttgcgccctcgccggcagcaagaagccacggaagt

ccgcccggagcagaaaatgcccacgctactgcgggtttatatagacggtccccacgggatggggaaaaccaccacca
cgcaactgctggtggccctgggttcgcgcgacgatatcgtctacgtacccgagccgatgacttactggcgggtgctg
ggggcttccgagacaatcgcgaacatctacaccacacaacaccgcctcgaccagggtgagatatcggccggggacgc
ggcggtggtaatgacaagcgcccagataacaatgggcatgccttatgccgtgaccgacgccgttctggctcctcata
tcgggggggaggctgggagctcacatgccccgcccccggccctcaccctcatcttcgaccgccatcccatcgccgcc
ctcctgtgctacccggccgcgcggtaccttatgggcagcatgacccccaggccgtgctggcgttcgtggccctcat
cccgccgaccttgcccggcaccaacatcgtgcttggggcccttccggaggacagacacatcgaccgcctggccaaac
gccagcgccccggcgagcggctggaactggctatgctggctgcgattcgccgcgtttacgggctacttgccaatacg
gtgcggtatctgcagtgcggcgggtcgtggcgggaggactggggacagctttcggggacggccgtgccgccccaggg
tgccgagccccagagcaacgcgggcccacgaccccatatcggggacacgttatttaccctgtttcgggcccccgagt
tgctggcccccaacggcgacctgtataacgtgtttgcctgggccttggacgtcttggccaaacgcctccgttccatg
cacgtctttatcctggattacgaccaatcgcccgccggctgccgggacgccctgctgcaacttacctccgggatggt
ccagacccacgtcaccacccccggctccataccgacgatatgcgacctggcgcgcacgtttgcccgggagatggggg
aggctaac**TGA**GCGGGACTCTGGGGTTCGCGAAATGACCGACCAAGCGACGCCCAACCTGCCATCACGA
GATTTCGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGG
ATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCCAACTTGTTTATTGCAGCTTAT
AATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGT
TGTGGTTTGT**CCAAACTCATCAATGTATCTTATCATGTCTG**AAGCTTataacttcgtatagtatacatta
tacgaagttat**GCTAGG**AAGGACCTGTCTGTCGCTGGGCTGTGGGTGCTAAGGGTAAGGGAGAGGGAGGGAGCCGGG
AGCCGTAGAGGGTGGCCGACGGCGGCGGCCCTCAAAAGCACTTGTTCCTTCTGCTTCTCCCTGGCTGACCCCTGGCC
GGCCCAGGCCTCCACGGGGGCGGCAGGCTGGGTTCATTCCCCGGCCCTCCGAGCCGCGCCAACGCACGCAACCCTTG
CTGCTGCCCGCGCGAAGTGGGCATTGCAAAGTGCGCTCATTTTAGGCCTCCTCTCTGCCACCACCCCATAATCTCAT
TCAAAGAATACTAGAATGGTAGCACTACCCGGCCGGAGCCGCCCACCGTCTTGGGTCGCCCTACCCTCACTCAAGTC
TGTCTGCCTCTCAGTCTCTTACCACCCCTCCTCCAATGTGATTCAATCCAATGTTTGGTCTCTCAGCGCTTACTCCC
CTTGCCTTGCTCCAAAGACGCTGCCGATCTGCTCTACTCCCAATCAGGTCCGGGATTTCAGGGCGCCTCACTCTGCC
TTAAAGCCACGAAGGCGACCCTCTGCCTTCTCCTCGTGCACTTTTCGGAGCCATTGCCCTCCCGGGGCGGAAGACCA
GGCTGTGAACTGGGAAAGCGCTAGCCCGGCCAGGGAGCATCTCCCCAGCCTCCCTGCGAACTGCGCCTGAAACGTGA
GCTGCGCTGCAGGTGCCTGGAGCACCGCGCATCTTTTTTTTTTAAATCTGTTTGTAAATTATATGATGCCTTTTGAA
ATCAATTTTGGTACAG**atgcat**

10 CURRICULUM VITAE

Personal Information

Surname: Cai

Name: Qing

Address: Rubensstraat 51, 2812 Muizen (Mechelen)

Mobile number: 0479/35.23.28

Place of birth: Tienen

Date of birth: 25/09/1983

Nationality: Belgian

Sexe: Female

E-mail address: qing.cai.be@gmail.com

Driving Licence: B

Education

2005 – 2007	Master in Bio-engineering Cell and Genes Biotechnology, KU Leuven Minor: Nanoscience and -nanobiotechnology
2001 – 2005	Master in Industrial Science of Chemistry option Biochemistry, Groep T, Leuven
2003 – 2004	Spanish Class, Ace Groep T Leuven
1997 – 1999	Chinese Class, Chinese School in Brussels
1995 – 2001	Secondary Education, Sciences and Mathematics Koninklijk Atheneum Tienen

Professional Experience

2013 – now Life Science Technology Researcher

Imec, Leuven

2007 – 2013 **PhD:**

Stamcelinstituut Leuven

(promoter prof. Verfaillie and copromoter prof. Mathieu)

"Prospectively isolated human NGN3-expressing progenitors give rise to pancreatic endocrine cells"

2006 - 2007 **Master thesis bio-engineering:**

Stamcelinstituut Leuven *(professor Verfaillie)*

"Characterization of endoderm differentiation cultures from multipotent adult progenitor cells."

2005 **Student Job:**

Lab technician at Corus Aluminium in Duffel.

2004 -2005 **Master thesis industrial engineering:**

Orafti in Tienen *(mentor: Bart Levecke)*

"Research in the enzymatic esterification of carbohydrates, inulin in particular."

Student Job:

"Jongeren en Media" as a representative in Brussels.

1994 – 2008 **Weekend Job:**

Family restaurants in Tienen and Gent

Grants

IWT (Agentschap voor innovatie door wetenschap en technologie)

1st term: January 2008 – December 2009

2nd term: January 2010 – December 2012

ISSCR

Travel award (June 2010)

Publications

Cai, Q., Bonfanti, P*., Sambathkumar, R.*, Vanuytsel, K., Vanhove, J., Gysemans, C., Debiec-Rychter, M., Raitano, S., Heimberg, H., Ordovas L.** and Verfaillie C.** (accepted Stem Cells Translational Medicine) *, **These authors contributed equally. **Prospectively isolated human NGN3-expressing progenitors give rise to endocrine hormone-expressing cells**

Kumar, A., Lo Nigro, A., Gysemans, C., Cai, Q., Heremans, Y., Esguerra, C., Abdul, M., Bansal-Pakala, M., Hering, B., Mathieu, C., Binas, B., Prósper, F., Verfaillie, C.M. and Barajas, B. PLoS One. 2013 May 9;8(5):e63491. doi: 10.1371/journal.pone.0063491. **Reversal of hyperglycemia by insulin-secreting cells derived from rat multipotent adult progenitor and extraembryonic endoderm precursor cells**

Poster presentations

Qing Cai, Laura Ordovas, Paola Bonfanti, Conny Gysemans, Kim Vanuytsel, Jolien Vanhove, Susanna Raitano, Wendy Vandendries, Harry Heimberg and Catherine M. Verfaillie. **Generation and specific isolation of pancreatic progenitor cells from human embryonic stem cells** Knowledge for growth, May 28 2012, Gent, Belgium (poster)

Qing Cai, Laura Ordovas, Kim Vanuytsel, Yonsil Park, Chantal Mathieu and Catherine Verfaillie. **Differences between 2D and 3D protocol in human ESCs towards pancreatic enoderm elucidated** ISSCR, June 15-18 2011, Toronto, Canada (poster)

Qing Cai, Tineke Notelaers, Chantal Mathieu and Catherine Verfaillie. **Differentiation of human ESCs into pancreatic progenitor and insulin-like positive cells** ISSCR, June 16-19 2010, San Fransisco, USA (poster and travel award)

Qing Cai, Chantal Mathieu and Catherine Verfaillie. **Generation and characterization of functional β-cells through differentiation of pluirpotent stem cells** BetaCellTherapy Training course, May 25-27 2009, Gent, Belgium (poster)

Miguel Barajas, Qing Cai, Camila V. Esguerra, Conny Gysemans, Bart Denys, Yves Heremans, Molly Nelson-Holte, Veerle Vanslembroek, Daniel W. Fraga, Mohamed Abdul, Pratima Bansal-Pakala, Bernhard Hering, Daniel Pipeleers, Chantal Mathieu and Catherine M. Verfaillie. P**roduction of pancreatic hormone-producing endocrine cells from rat multipotent adult progenitor cells.** BetaCellTherapy Training course, May 28-30 2008, Gentofte, Denmark (poster)